5-2009

A PULSATILE BIOREACTOR FOR CONDITIONING TISSUE ENGINEERED HEART VALVES

Leslie Sierad
Clemson University, sierad@alum.wpi.edu

A PULSATILE BIOREACTOR FOR CONDITIONING TISSUE ENGINEERED HEART VALVES

A Thesis
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Bioengineering

---

by
Leslie Neil Sierad
May 2009

Accepted by:
Dr. Dan T. Simionescu, Committee Chair
Dr. Jiro Nagatomi
Dr. Agneta Simionescu
Dr. Ken Webb

ABSTRACT

Tissue engineered constructs with autologous adult stem cells capable of self-repair and growth are highly desired replacements for diseased heart valves. However, the current approaches have inadequate mechanical properties to withstand *in vivo* implantation. Therefore, our group hypothesized that an *in vitro* environment of physiological intra-cardiac pressures and flow will stimulate stem cells to differentiate and remodel valvular scaffold constructs before implantation.

The group developed a pneumatic-driven conditioning system (Aim I) consisting of a three-chambered heart valve bioreactor, a pressurized compliance tank, a reservoir tank, one-way valves, pressure-retaining valves, and pressure transducers. The system can be sterilized using conventional autoclaving and ethylene oxide gas. The most novel feature is its ability to accommodate all clinically relevant sizes of stented or stentless biological, mechanical, or tissue engineered substitutes. A tissue derived heart valve substitute was used to test the bioreactor's functional capabilities (Aim II) at 60 beats per minute. The tests resulted in excellent opening and closing of the valve, pulsatile flows reaching 1400 mL per minute, and aortic pressures reaching 100 mmHg. The bioreactor then tested tissue engineered heart valves (Aim III) made from decellularized and lightly cross-linked tissues. Two stentless porcine aortic heart valves were conditioned in the bioreactor for 21 days. The first was seeded with adipose-derived stem cells (valve 1) and the second with aortic endothelial cells (valve 2). The third valve was made of valve-shaped fibrous sheets encasing a spongy collagen scaffold. It was seeded with human

bone marrow-derived stem cells (valve 3) and conditioned in the bioreactor for 8 days. After progressive adaptation, valves were tested at 60 beats per minute and 10 mL per stroke. Each experiment also included a static control.

The bioreactor created proper closing and opening of the heart valves and allowed for multiple mounting methods. Results indicated successful cell seeding and attachment in valves 1, 2, and 3; noticeable intercellular alignment in valves 2 and 3; and stem cell differentiation in valve 3. Overall, the conditioning system provides a dynamic three-dimensional cell culture setting designed to provide optimal physiological conditions for tissue engineered heart valve development over extended time periods. The group will continue to develop this approach to study multiple aspects of tissue engineered heart valve development and heart valve pathology.

DEDICATION

I would like to dedicate this thesis to my Lord and Savior, Jesus Christ. Only by His grace have I been able to study at Clemson and complete this research.

Through the law came the knowledge of my sin and the realization that I needed to be saved from the penalty of those sins. Were it not for His perfect life given as an example, His death as a substitutionary payment for my sins, His resurrection as victory over death, and His offer of redemption as salvation for my soul, I would have no hope for my future. But by His grace, I have agreed with Him about the circumstances of my life and have accepted His gift of salvation through faith alone. Therefore, I have a living hope for my future and a purpose for my life that is far greater than any other: to bring glory to God.

It is for this purpose that I live, and it is for this purpose that I am performing research at Clemson University. As such, I dedicate this work to the Lord Jesus Christ, without whom I would have neither the strength nor the skills to complete it.

And there is no other God besides Me,
A righteous God and a Savior;
There is none except Me.
Turn to Me and be saved, all the ends of the earth;
For I am God, and there is no other.
~Isaiah 45:21b-22~

ACKNOWLEDGEMENTS

My deepest appreciation is owed to Dr. Dan Simionescu for the endless support and guidance extended every step of the way. I could not ask for a more encouraging advisor. I have been blessed to be guided by an advisor that is not only focused on research but is also concerned with and greatly invested in the growth of the students.

I wish to thank all the members of the Biocompatibility and Tissue Regeneration Laboratory. Many pieces of this project would not be possible without the collaborative experiments of Betsy Tedder, Chris Albers, Dr. Agneta Simionescu, and Dr. Dan Simionescu. Their willing investment of time and research was vital and is much appreciated. Thanks to Tom Chuang and Jeremy Mercuri for their aid and expertise in our research. This was truly a team effort of which I am extremely grateful.

My gratitude is extended to my committee members: Dr. Charles Webb, Dr. Jiro Nagotomi, Dr. Agneta Simionescu, and Dr. Dan Simionescu. Their leadership, expertise, and insight are invaluable.

Thanks to Clemson University, the Bioengineering department, and the NIH and INBRE for the opportunity to conduct research and the funding making this project possible. The Clemson University Machining and Technical Services deserves much appreciation for superbly manufacturing the bioreactor along with the Electron Microscope Facility for their assistance in acquiring images.

My sincere appreciation goes to Theresa Vosburgh for the many hours spent editing with such short notice. Thanks to Snow Creek Meat Processing plant for their generous donation of heart valves and tissues.

TABLE OF CONTENTS

Page

ABSTRACT i

DEDICATION iii

ACKNOWLEDGEMENTS iv

LIST OF TABLES viii

LIST OF FIGURES ix

CHAPTER 1: INTRODUCTION AND BACKGROUND 1

1.1 Cardiac Anatomy & Physiology 1

1.1.1 The Heart as a Pump 2

1.1.2 Aortic Valve Anatomy and Physiology 3

1.1.3 Aortic Valvular Disease 5

1.2 Heart Valve Bioreactors 6

1.2.1 Bioreactor Requirements 6

1.2.2 Current Bioreactors 7

CHAPTER 2: PROJECT APPROACH 10

2.1 Overview 10

2.2 Specific Aims 11

2.2.1 Aim I: Design and Build a Heart Valve Bioreactor 11

2.2.2 Aim II: Test the Heart Valve Bioreactor's Capabilities as a Pump 11

2.2.3 Aim III: Use the Heart Valve Bioreactor to Test Tissue Engineered Heart Valves 12

CHAPTER 3: MATERIALS AND METHODS 13

3.1 Bioreactor Design 13

3.1.1 Customer and Design Goals (Who, What, & Who vs. What) 13

3.1.2 Current Devices' Capabilities (Now & Now vs. What) 16

3.1.3 Measurement, Evaluation, and Desired Values (How, How vs. What, & How Much) 16

Table of Contents (Continued)

Page

3.1.4 Synergism and Conflictions (How vs. How) 17
3.2 Bioreactor Capabilities Testing Using a Bioprosthetic Heart Valve 18
3.2.1 Pressure 18
3.2.2 Flow 20
3.3 Experimental Methods 21
3.3.1 Porcine Aortic Valve Collection and Cleaning 21
3.3.2 Tissue Decellularization 21
3.3.3 Tissue Fixation 22
3.3.4 Neutralization and Cellular Seeding 23
3.3.5 Live/DEAD® staining 23
3.3.6 MTS 24
3.3.7 Diff-Quick 25
3.3.8 Scanning Electron Microscopy 25
3.3.9 Hematoxylin and Eosin Sections 25
3.3.10 DAPI Sections 26
3.4 Heart Valve Experimentation 28
3.4.1 Valve 1: Living Valve – 7 Days 28
3.4.2 Valve 2: Endothelial Cell Seeded Valve – 21 Days 29
3.4.3 Valve 3: Adipose-derived Stem Cell Seeded Valve – 21 Days 32
3.4.4 Valve 4: Tri-layered Tissue Engineered Heart Valve – 8 Days 33

CHAPTER 4: RESULTS 37
4.1 Bioreactor Design 37
4.2 Bioreactor Capabilities Testing Using a Bioprosthetic Heart Valve 40
4.2.1 Pressure 40
4.2.2 Flow 43
4.3 Heart Valve Experimentation 45
4.3.1 Valve 1: Living Valve – 7 Days 47
4.3.2 Valve 2: Endothelial Cell Seeded Valve – 21 Days 49
4.3.3 Valve 3: Adipose-derived Stem Cell Seeded Valve – 21 Days 59
4.3.4 Valve 4: Tri-layered Tissue Engineered Heart Valve – 8 Days 60

CHAPTER 5: ANALYSIS AND DISCUSSION 62

Table of Contents (Continued)

Page

5.1 Bioreactor Design 62
5.2 Bioreactor Capabilities Testing Using a Bioprosthetic Heart Valve 63
5.3 Heart Valve Experimentation 64

CHAPTER 6: CONCLUSIONS 67

CHAPTER 7: RECOMMENDATIONS 68

APPENDICES 69
Appendix A: Design Documentation 70
Appendix B: Digitally Inverted DAPI Images 82

REFERENCES 83

LIST OF TABLES

Table Page

Table 1: Composition and function of each valve layer. 4

Table 2: Experimental conditions & assays for living valve experiment. 29

Table 3: Experimental conditions & assays for endothelial cell experiment..... 31

Table 4: Experimental conditions & assays for stem cell experiment..... 33

Table 5: Experimental conditions & assays for tri-layered tissue engineered heart valve..... 36

Table 6: Stroke volumes at 60 beats per minute 43

Table 7: Optical density of culture solution via MTS assay. 48

Figure 26: SEM images from the endothelial cell experiment, 21 days, cells, static. 56

Figure 27: SEM images from the endothelial cell experiment, 21 days, cells, dynamic..... 57

Figure 28: SEM images from the endothelial cell experiment, 21 days, cells, dynamic..... 58

LIST OF FIGURES

Figure Page

Figure 1: Blood flow through the heart 1

Figure 2: The cardiac cycle for the left heart 3

Figure 3: Aortic valve anatomy. 4

Figure 4: Schematic diagram of a model pulsatile flow bioreactor 9

Figure 5: Objectives tree 15

Figure 6: Assembly of bioreactor system for pressure testing. 19

Figure 7: Cross sectional view of the tri-layered leaflet. 35

Figure 8: Formation of the tissue engineered heart valve 35

Figure 9: Culture system schematic 37

Figure 10: Picture and computer aided drafting representation of assembled bioreactor. 39

Figure 11: Cross-sectional view of the bioreactor demonstrating the pumping actions 39

Figure 12: Chamber pressures 41

Figure 13: Chamber pressures without external application of pressure 42

Figure 14: Chamber pressures with external application of pressure 42

Figure 15: Stroke volume for multiple settings of the ventilator 44

Figure 16: Total fluid flow through the valve per minute. 44

Figure 17: Comparison of all experimental valves. 46

Figure 18: Results of the living valve experiment, 7 days. 48

Figure 19: Live/DEAD staining solution. 49

Figure 20: Results of the endothelial cell experiment, 0 days. 50

List of Figures (Continued)

Figure Page

Figure 21: Results of the endothelial cell experiment, 21 days. 51

Figure 22: SEM images from the endothelial cell experiment,
0 days, no cells, static. 52

Figure 23: SEM images from the endothelial cell experiment,
0 days, cells, static. 53

Figure 24: SEM images from the endothelial cell experiment,
0 days, cells, static. 54

Figure 25: SEM images from the endothelial cell experiment,
21 days, cells, static. 55

Figure 26: SEM images from the endothelial cell experiment,
21 days, cells, static. 56

Figure 27: SEM images from the endothelial cell experiment,
21 days, cells, dynamic 57

Figure 28: SEM images from the endothelial cell experiment,
21 days, cells, dynamic 58

Figure 29: Results of the adipose-derived stem cell experiment, 21 days. 59

Figure 30: Actin vs. vimentin in valvular insterstitial cells for comparison. 60

Figure 31: Results of the tissue engineered heart valve experiment, 8 days. 61

CHAPTER 1: INTRODUCTION AND BACKGROUND

1.1 Cardiac Anatomy & Physiology

The heart has two double-chambered pumps. Figure 1 demonstrates the pressures and flow through these chambers. The right pump pushes blood through the lungs, where waste gases are exchanged for nutritional gasses. The left pump propels blood through the peripheral organs. Each group has two types of chambers. In each case, blood first enters the superior chamber, the atrium, which helps move blood through an atrioventricular valve into the ventricle. When these ventricles contract, blood propels through the pulmonary (right side) or ventricular (left side) valve and through the rest of the body. Following contraction, the pulmonary and aortic valves close to prevent blood flow back through the heart (Guyton and Hall, 2006).

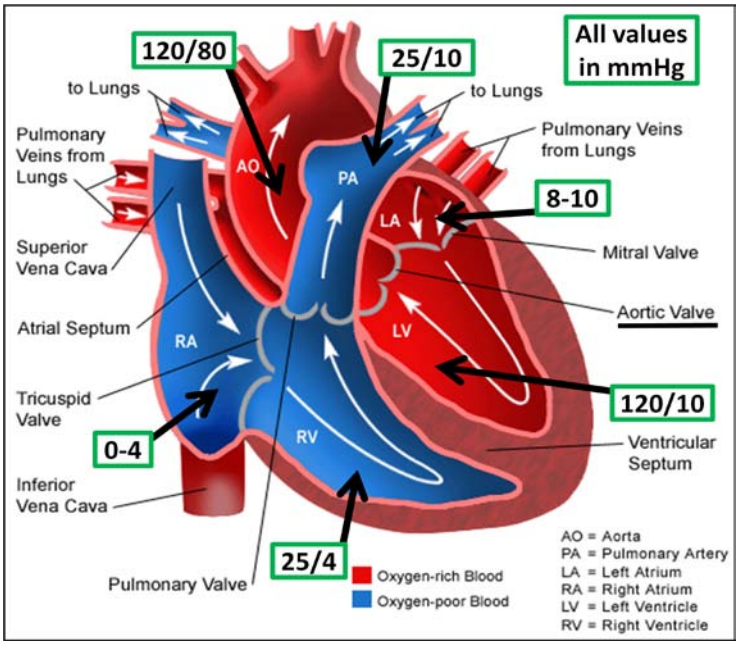


Figure 1: Blood flow through the heart. (Darling, ; Guyton and Hall, 2006)

This cycle of opening and closing, sudden changes of pressure, and various other mechanical stresses make heart valves the most mechanically stressed tissues in the body (Bilodeau and Mantovani, 2006). This is especially true for the aortic valve, where the pressures are very high compared to the forces on the rest of the valves. These stresses often cause damage to the valves, in the form of microtears and calcific deposits. Since the aortic valve is the most stressed, it follows that it is also the most often diseased and replaced. Therefore, this project has focused on the aortic (Guyton and Hall, 2006).

1.1.1 The Heart as a Pump

The pumping heart is a dynamic feedback system that changes its physical and chemical outputs according to the body's needs. However, under average normal resting conditions, the left heart beats about 70 times per minute (bpm), ejecting 70 mL (stroke volume) of its left ventricular volume of 120 mL (end-diastolic volume) each beat. This results in a total of about 4.9 liters of blood flow per minute. The ventricles' cyclic contractions create pressures that drive the blood through the circulatory system. When the left ventricle contracts, the pressure increases to about 120 mmHg. At this point, the aortic valve snaps shut and blood flows away from the heart along the pressure gradient. By the time the pressure in the aorta drops to about 80 mmHg, the left ventricle has begun its contraction to pump another bolus of blood and return the pressure to 120 mmHg. Figure 2 shows the left ventricular volumes and pressures as described above (Guyton and Hall, 2006).

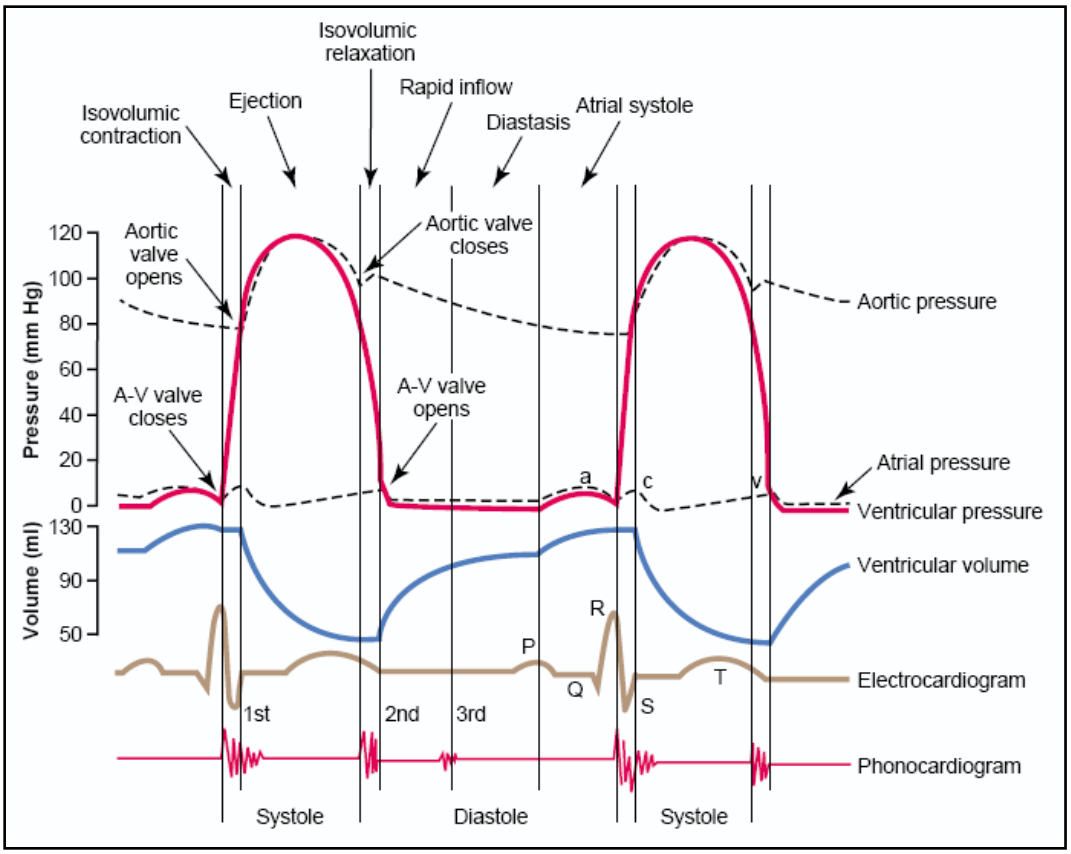


Figure 2: The cardiac cycle for the left heart. (Guyton and Hall, 2006)

1.1.2 Aortic Valve Anatomy and Physiology

The physiological composition of a human aortic heart valve is optimized to withstand high physical stresses and respond to pressure changes during diastolic and systolic stages of the cardiac cycle. The tricuspid valve is constructed with very strong, yet very pliable fibrous tissue with three heterogeneous leaflets attached to an annular ring (Fong, Shin'oka et al., 2006). These leaflets open during systole and press against the aortic root wall. When they snap close at the start of diastole they meet to create a seal that can prevent backflow even with the 100 mmHg transvalvular pressure difference. This cycle maintains efficient unidirectional flow with each heart beat.

Valve leaflets consist of three layers of tissue that together have the necessary biomechanical properties to withstand cyclic loading. Table 1 displays each layer's composition and function. Figure 3 shows a view of the aortic valve and a cross-sectional view of a cusp.

Table 1: Composition and function of each valve layer.

Layer		Matrix Composition	Function
Top	Fibrosa	Densely packed circumferencially aligned collagen; elastin	Maintain durability and stiffness to withstand 80 mmHg pressure gradient
Middle	Spongiosa	Loosely arranged collagen; glycosaminoglycans	Allows shearing between the ventricularis and the fibrosa during valve cycle
Bottom	Ventricularis	Densely packed collagen; radially aligned elastin	Maintains valve resilience, allows stretch in response to pressure changes

(Vesely, 2004; Schoen, 2005; Simionescu, 2006)

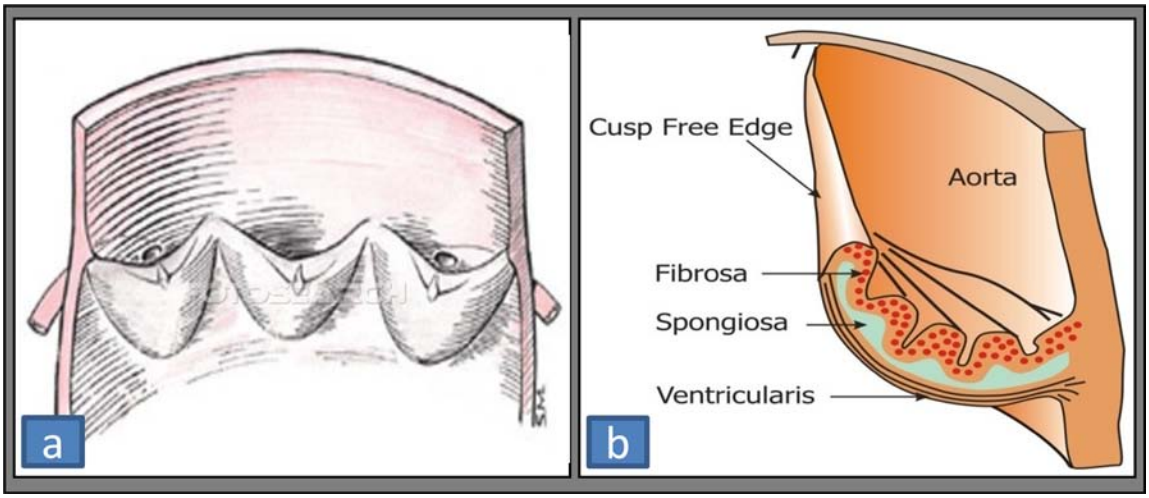


Figure 3: Aortic valve anatomy. a) aortic valve dissected for viewing (LifeART). b) cross section of a cusp, showing the three layers present (Vesely, 1998).

1.1.3 Aortic Valvular Disease

Aortic valvular heart disease is classified into two main types: 1) stenosis or 2) regurgitation. There are various causes for each of these problems, but the final outcome is the same. Patients suffering from valvular heart disease will eventually need a heart valve replacement (Guyton and Hall, 2006).

In aortic stenosis, the left ventricle fails to empty adequately following contraction because the aortic valve does not open properly, creating a reduction in the valvular orifice. In aortic regurgitation, blood flows back into the left ventricle after contraction because the aortic valve does not close properly (Guyton and Hall, 2006).

Valvular heart disease can be a congenital defect such as improper valve size, malformed leaflets, or an irregularity in the way the leaflets are attached. It may also be caused by damage acquired after birth such as calcific deposits, changes in the structure of the valve, or infections like infective endocarditis and rheumatic fever. Both stenosis and regurgitation lead to a reduction in the net stroke volume output of the heart, causing it to work harder to pump the same amount of blood through the body (Clinic). Eventually, the diseased valve must be replaced.

1.2 Heart Valve Bioreactors

Bioreactors can be defined "as devices in which biological and/or biochemical processes develop under closely monitored and tightly controlled environmental and operating conditions (e.g. pH, temperature, pressure, nutrient supply, and waste removal)" (Martin, Wendt et al., 2004). Requirements for such bioreactors and examples of previous aortic valve bioreactors are discussed below.

1.2.1 Bioreactor Requirements

Freed (Freed and Vunjak-Novakovic, 2000) says that a bioreactor must be able to perform at least one of the following five functions: (1) establish a uniform distribution of cells on a three-dimensional (3D) scaffold, (2) maintain the desired concentration of gases and nutrients in the culture medium, (3) provide efficient mass transfer to the growing tissue, (4) expose developing tissue to physical stimuli, and (5) provide information regarding the formation process of 3D tissues, which originate from the isolated cells. This project focused on functions 2, 3, and 4 of Freed's declared functions.

One of the most important controls of a bioreactor is to supply an adequate amount of oxygen to a 3D tissue construct. Other biochemical factors, such as carbon dioxide and wastes also require adequate transport. In conventional cell culture, this is most often done by creating a large liquid to sterile air interface to facilitate the diffusive transport of oxygen. In a bioreactor, where the volume of culture medium present is much larger than in standard cell culture, additional methods must be used. These methods range from a sterile filter open to the external environment in combination with flow of

the culture medium (Dumont, Yperman et al., 2002) to coiling gas-permeable tubing inside a culture medium reservoir (Warnock, Konduri et al., 2005).

Physical stimuli such as tension, compression, shear stresses, pressure, temperature, and pulsatile flow of culture medium improve the structure and mechanical properties of engineered tissues (Rabkin and Schoen, 2002; Barron, Lyons et al., 2003). These mechanical forces have an integral part in regulation of cell phenotype and growth and the repair or degradation of tissues (Rabkin and Schoen, 2002). Control of trans-valvular pressure, pulsatile forces, flow rate, frequency, stroke rate, and stroke volume are all important design parameters of bioreactor to ensure that the necessary physical stimuli are integrated into the design (Barron, Lyons et al., 2003).

1.2.2 Current Bioreactors

Current heart valve bioreactors are designed to test a single valve *in vitro* at different levels of pulsatile flow under controlled conditions in a standard humidified incubator at 37°C and 5% CO_2. Figure 4 shows the common main components of aortic valve bioreactors, which include a reservoir, mechanical valve, pump, processing chamber, compliance chamber, resistance, and a filter (Barron, Lyons et al., 2003). This schematic gives one representative layout of these components, but many bioreactors in use today have variations on placement, type, and extent of use of these components. Most bioreactors have control over parameters such as flow, pH, and stroke volume and can change those parameters as desired to create varying environments for the valves. One use of the controllable parameters is progressive adaptation, where increasing

amounts of mechanical stimuli are applied to aid strengthening of the tissue construct and prevent immediate failure upon implantation.

There are currently numerous individual laboratories across the country using heart valve bioreactors. Many reviews (Freed and Vunjak-Novakovic, 2000; Rabkin and Schoen, 2002; Ratcliffe and Niklason, 2002; Barron, Lyons et al., 2003; Martin, Wendt et al., 2004; Martin and Vermette, 2005; Bilodeau and Mantovani, 2006) contain extensive comparison and assessment of the variations between them. The bioreactors range in design, complexity, and function, performing at various levels and accuracy. Valve mounting methods and modes of assembly vary, often with little options or ability to accommodate abnormal valve shapes. While many bioreactors are very powerful and can achieve high pressurization, none have been found to be able to subject valves to the wide range of synergistic physiological stresses that would be present if the valve were implanted into the body (Bilodeau and Mantovani, 2006).

Overall, much progress has been made in the area of heart valve bioreactors. However, there is still room for improvement.

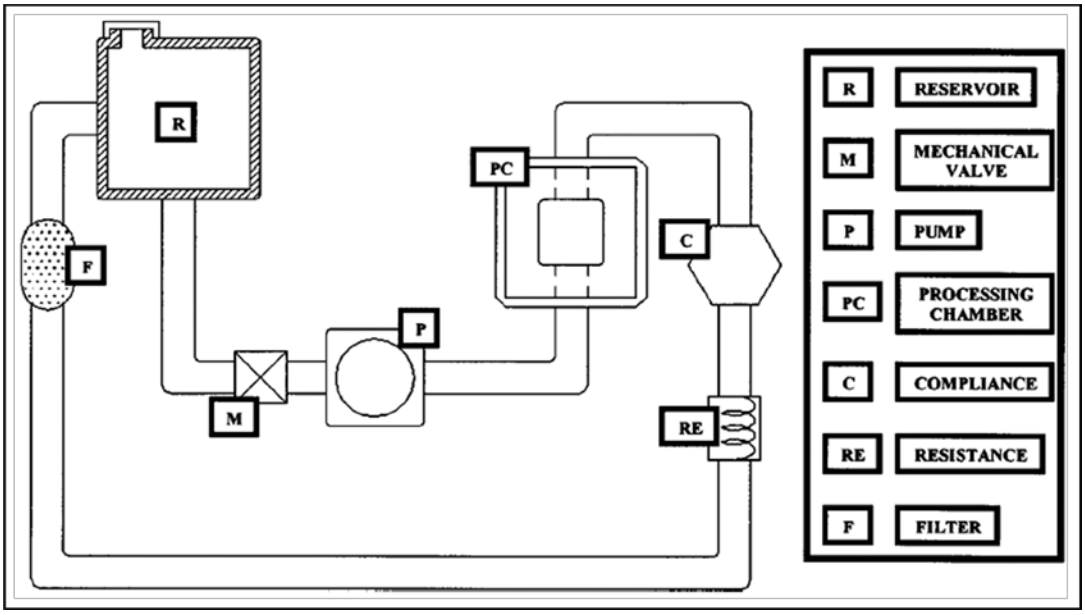


Figure 4: Schematic diagram of a model pulsatile flow bioreactor. (Barron, Lyons et al., 2003)

CHAPTER 2: PROJECT APPROACH

2.1 Overview

Existing heart valve replacements provide major improvements in cardiac function and life expectancy but have significant limitations and require either lifelong anticoagulation therapy for the mechanical valves or surgical replacement within 15-20 years for tissue derived valves. Thus, tissue engineered constructs with autologous adult stem cells capable of self-repair and growth are highly desired replacements for diseased heart valves. In response to the current approaches' inadequate mechanical properties upon *in vivo* implantation, our group hypothesizes that an *in vitro* environment of physiological intra-cardiac pressures and flow will stimulate stem cells to differentiate and remodel valvular scaffold constructs before implantation.

The goal of this master's project has been to develop, build, and test a pulsatile bioreactor that will cause a valve to cyclically open and close and will provide the physiological intra-cardiac pressures and flow to condition tissue engineered heart valves.

2.2 Specific Aims

This work has three distinct parts: 1) designing and building a heart valve bioreactor, 2) testing the heart valve bioreactor's capabilities as a pump, and 3) using the heart valve bioreactor to test tissue engineered heart valves.

2.2.1 Aim I: Design and Build a Heart Valve Bioreactor

The desired heart valve bioreactor had three fundamental functional requirements: That 1) the valve open and close cyclically due to a change in trans-valvular pressure, 2) the pressure be adjustable to simulate intra-cardiac pressures, and 3) the fluid in the culture system circulate to achieve nutrient and waste transport and create appropriate sheer stresses on the valve surface. All materials were to be non-toxic and non-degradable. The system was to maintain sterility and visibility of the operation of the heart valve.

Evaluative design methods provided the basis for the bioreactor's systematic design. First, we identified the customers and their wants. Then we identified and evaluated the competition and developed means to evaluate the customers' wants. After setting minimum and optimal values for each desired capability, we identified any conflicting effects of improving outcomes. After completing the design, the drawings were drafted, the manufacturing was accomplished, and the system was tested.

2.2.2 Aim II: Test the Heart Valve Bioreactor's Capabilities as a Pump

The desired environmental conditions of the bioreactor as a pump include 1) aortic systolic/diastolic pressures reaching at least 120/80 mmHg, 2) flow rates reaching

5000 mL/min, 3) stroke rate exceeding 60 beats per minute, 4) oxygen and carbon dioxide levels high enough to support cell and tissue growth, and 5) repeatable conditions.

A bioprosthetic heart valve was used to test the conditioning system under both light and rigorous conditions. We used flow meters and pressure transducers to evaluate the bioreactor's capabilities as a pump in according to the desired conditions.

2.2.3 Aim III: Use the Heart Valve Bioreactor to Test Tissue Engineered Heart Valves

The desired use of this bioreactor is to create an *in vitro* environment of physiological intra-cardiac pressures and flow for the conditioning and testing of tissue engineered heart valves.

The system's ability to sustain a freshly collected, living heart valve (valve 1) was first tested. After that, the bioreactor tested tissue engineered heart valves made from decellularized and lightly cross-linked tissues. Two stentless porcine aortic heart valves were tested in the bioreactor for 21 days. The first was seeded with rat adipose-derived stem cells (valve 2) and the second with porcine aortic endothelial cells (valve 3). The last valve was made of valve-shaped fibrous sheets encasing a spongy collagen scaffold. It was seeded with human bone marrow-derived stem cells (valve 4) and conditioned in the bioreactor for 8 days.

Cellular assays including Live/DEAD, MTS, Diff-Quick, SEM, H&E staining, and DAPI staining were used to evaluate cell viability and stem cell differentiation.

CHAPTER 3: MATERIALS AND METHODS

3.1 Bioreactor Design

The bioreactor's development was approached as a design project. As such, certain formal methods of design were used to determine the desired qualities of the system and those qualities' desired values. The design process closely followed Dym and Little's (2004) design book Engineering Design: A Project-Based Introduction. These methods can also be documented using a quality function development (QFD) chart. The QFD development process can be found in The Mechanical Design Process by Ullman (2003). The following subsections detail the use of these design processes.

3.1.1 Customer and Design Goals (Who, What, & Who vs. What)

A fundamental step in any design project is to identify the customers, then move on to what the customers desire (attributes) from the device. The primary customer for this project was the principle investigator of the Biocompatibility and Tissue Regeneration Laboratory (BTRL), Dr. Dan Simionescu. The initial client statement provided was to "Create a bioreactor to test and condition about 10 replacement heart valves."

Expansion of the project revealed additional customers in the form of other lab members. These included Mary E. "Betsy" Tedder, Ting-Hsien "Tom" Chuang, and Dr. Agneta Simionescu. Through interviews, a customer questionnaire, and a review of current devices, a comprehensive design attributes list was compiled and divided into objectives, functions, and constraints. After pruning and grouping the objectives list, pair-

wise comparison charts were used to rank the importance of each objective for each user. These were combined to form a comprehensive ranked objectives list, which helped develop the objectives tree seen in Figure 5. Using these ranked objectives and a further understanding of the customers' desired attributes, an updated client statement was developed:

> "Create a bioreactor to simultaneously test and condition 3-6 replacement heart valves under physiological mechanical and nutritional conditions. The vital conditions of the aforementioned environment include cyclic opening and closing of the valve, aortic systolic/diastolic pressures reaching at least 120/80 mmHg and oxygen and carbon dioxide levels high enough to support the growth of the cells and tissues. Flow rates reaching 5000 mL/min and a stroke rate exceeding 60 beats per minute are also desired. This bioreactor should be consistent in its repeatability of conditions between cycles and over the life of the bioreactor. All materials used must be non-toxic and non-degradable. Sterility and visibility of the operation of the heart valves should be maintained."

The design was based upon this updated client statement and the previously mentioned ranking of objectives. Reviewing these items periodically ensured that the design continued to be what the customers desired.

The design documentation used to identify and develop the customers and objectives can be found in Appendix A: Design Documentation.

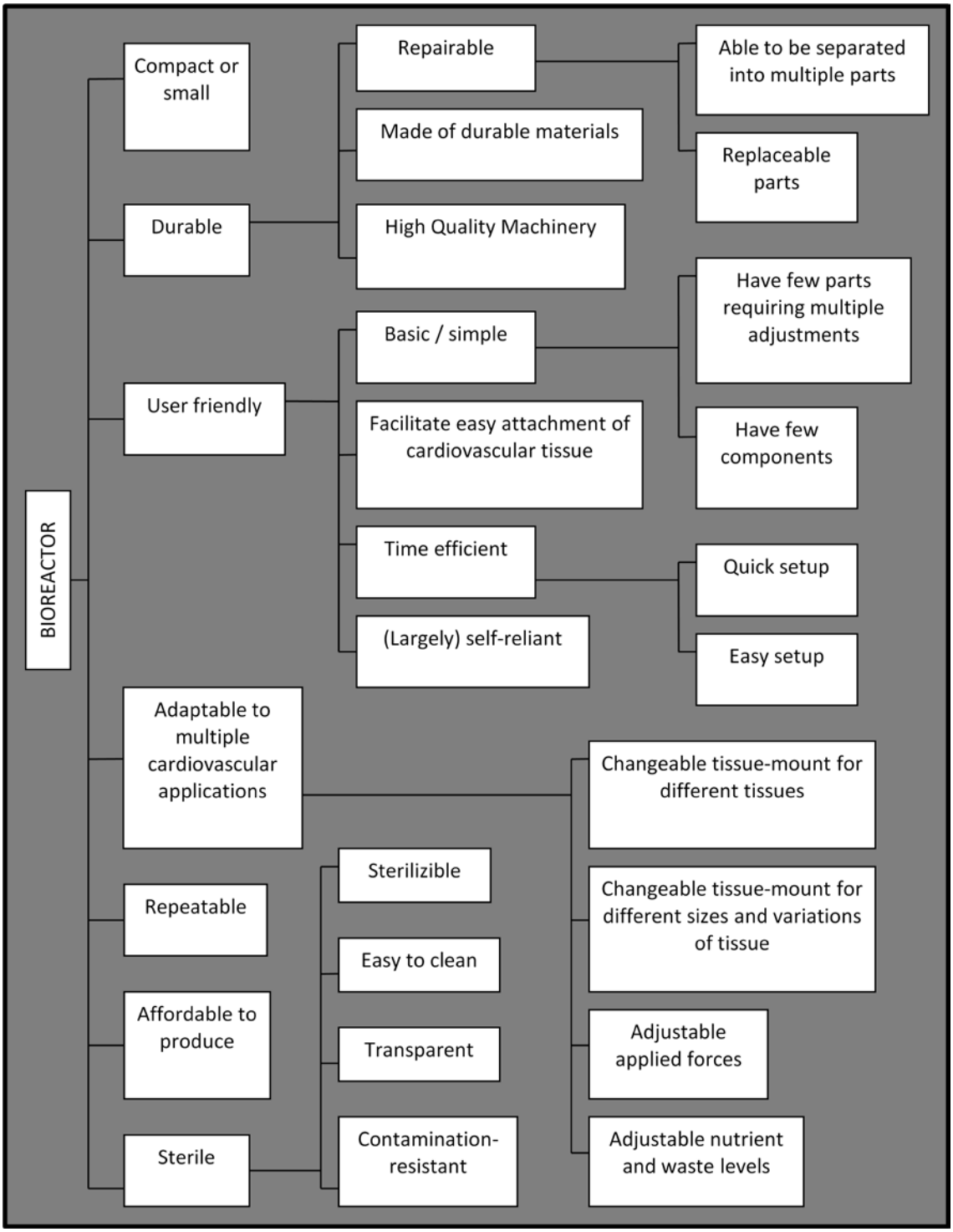


Figure 5: Objectives tree

3.1.2 Current Devices' Capabilities (Now & Now vs. What)

Section 1.2.2 described typical existing heart valve bioreactors. Some of these bioreactors were extremely similar and some were very different from the desired system. The desired attributes from all the designs were considered, but only the most closely related devices were further investigated. Every device was ranked for each of the design attributes. Those rankings then summarized the most effective means currently implemented to meet the desired attributes. After this evaluation, it was then possible to investigate methods of improvement for each design attribute as necessary.

3.1.3 Measurement, Evaluation, and Desired Values (How, How vs. What, & How Much)

The next step of the design process was to determine how each desired attribute would be measured (metrics) and the units in which those measurements could be recorded. Some were fairly obvious, such as desired pressure being measured in mmHg, while others were less obvious, such as sterilizability being measured in hours taken to sterilize completely.

After determining the metrics for each attribute, a range of acceptable values was developed. This range extended from levels at which it was expected that the customer would be delighted by the outcome to levels at which it was expected that the customer would be disgusted by the outcome. For example, for maximum attainable flow rate, disgusted and delighted values of 500 mL/min and 1000 mL/min, respectively were set. If the final design provided a maximum flow rate less than 500 mL/min, it was expected that the customers would not be satisfied. However, if the final design provided a

maximum flow rate of more than 1000 mL/min, it was expected that the customers would be fully satisfied.

3.1.4 Synergism and Conflictions (How vs. How)

Finally, to give a better understanding of the design, the ways in which improving one attribute would affect the other attributes was assessed. Some had positive interactions while others had negative interactions and a balance of improvements had to be made. More often, improving one attribute had no affect on the performance of other attributes.

3.2 Bioreactor Capabilities Testing Using a Bioprosthetic Valve

To demonstrate the achievement of the heart valve bioreactor's two fundamental functional requirements, "aortic" pressure, "ventricular" pressure, and fluid flow were tested. All tests used double-distilled water and a bioprosthetic heart valve (BHV). The BHV was an expired porcine glutaraldehyde fixed stented aortic valve replacement, 25mm Toronto SPV Valve from St. Jude Medical.

3.2.1 Pressure

The end diastolic- and end systolic- left ventricular and aortic pressure were determined by obtaining pressure readings from each of the aortic and ventricular chambers of the bioreactor. Pressure transducers (Cole-Parmer, K-68075-16) were mounted and a data acquisition system (DAQ) (OMEGA, OMB-DAQ-55) took readings at 180 Hz. The aortic pressure transducer was approximately 7.5 cm above the valve and the ventricular pressure transducer was approximately 8.0 cm below the valve. As per normal settings, the ventilator was set to have an inspiration time of 50%, pause time of 0%, working pressure of 110 cmH_2O, 60 bpm, and an actual inspiration tidal volume of 138±2mL. The total volume of fluid in the system was estimated to be 1,600 mL. This fluid was distributed between the bioreactor and two reservoirs: one to be pressurized and one to serve for gas exchange. The setup of the system can be seen in Figure 6.

Values of pressure were recorded under no pressurization, after an initial pressurization of the top reservoir using compressed air, and after a second pressurization of the top reservoir.

Figure 6: Assembly of bioreactor system for pressure testing.

3.2.2 Flow

Cyclic flow volume was determined using one flow meter (Cole-Parmer catalog #: K-32550-07) on each of the two outlet lines from the aortic chamber going to the reservoir. The top and bottom fluid levels with respect to the bottom of the bioreactor were 13cm and 9.5cm, respectively. As per normal settings, the ventilator was set to have an inspiration time of 50%, pause time of 0%, and working pressure of 108 cmH_2O.

Following one minute of cyclic flow at 60 beats per minute (bpm), the values were recorded to determine the flow per minute and stroke volumes attainable at these conditions. The actual inspiration tidal volume was adjusted while keeping all other variables constant to determine flow capabilities. Each level of actual inspiration tidal volume was repeated two times (n=2) and the readings averaged.

3.3 Experimental Methods

The following protocols were used in the experimentation, testing, and analysis of valves in the bioreactor.

3.3.1 Porcine Aortic Valve Collection and Cleaning

Fresh porcine hearts, with intact ascending aorta, were collected from a local USDA approved abattoir, Snow Creek Meat Processing, Seneca, SC. The aortic valves were dissected from the hearts in a section of tissue including the aortic root, aortic valve, adjacent portion of the mitral valve, and excess muscular tissue on the opposite side as the mitral valve. Valves were collectively stored in a 500mL bottle of un-buffered, sterile saline with 2% antibiotics/antimycotics over ice during transportation to the laboratory. The valves were then cleaned over ice on the lab bench. To clean the valves, the tissue was trimmed of excess fat, the aortic root was trimmed to a length of about one inch, the mitral valve was trimmed of excess material, and the muscle was trimmed to a thickness of less than one mm adjacent to the endocardium.

3.3.2 Tissue Decellularization

To minimize the immune responses, the porcine aortic valves were decellularized by removing cellular components and Gal-α (1,3) Gal, a porcine specific antigen. This was performed via steps of hypotonic shock using double distilled water (ddH_2O), extraction of cell fragments using 0.05M NaOH then decellularization solution (0.05% SDS, 0.5% Triton X-100, 0.5% Na-Deoxycholate, and 0.2% EDTA in 10mM TRIS, pH: 7.5), removal of nucleic acids using DNAse/RNAse, and sterilization using 70% EtOH.

Materials:

- Heart valves
- ddH_2O
- sterile ddH_2O
- 70% EtOH
- Sterile 1x PBS
- Sterile suture
- 10mM TRIS (2.42g TRIS in 2L ddH_2O at **pH:7.4-7.5**)
- 0.05 M NaOH (2g NaOH in 1L 10mM TRIS)
- Decellularization solution (in 1 Liter 10mM TRIS)
    - 0.05% SDS (Sodium Dodecyl Sulfate) (0.5g) – wear a breathing mask
    - 0.5% Triton X-100 (5 mL)
    - 0.5% Deoxycholic Acid, Sodium Salt (5g) – wear a breathing mask
    - 0.2% EDTA (Ethylenediaminetetra-acetic acid) (2g)
- DNAse/RNAse (deoxyribonuclease/ribonuclease) (in 1 Liter PBS)
    - 1.015g $MgCl_2$
    - 0.18mg DNAse (360 milliunits/ml)
    - 3.7mg RNAse (360 milliunits/ml)

Methods:

1. Rinse valves with ddH_2O
2. Incubate valves in ddH_2O **overnight** at *4°C*
3. Incubate valves in 0.05 M NaOH **2 hours** at *room temperature* under agitation
4. Rinse valves w/ddH_2O (3x)
5. Sterilize valves in 70% EtOH **20 minutes** at *room temperature* under agitation
6. Rinse valves w/ddH_2O (3x)
7. Incubate in decellularization solution **overnight** at *room temperature* under agitation
8. Rinse with ddH_2O (5-10x)
9. Sterilize valves in 70% EtOH **20 minutes** at *room temperature* under agitation
10. Rinse valves with ddH_2O (3x)
11. Incubate valves in ddH_2O **2 hours** at *room temperature* under agitation
12. Incubate in RNAse/DNAse **overnight** at *room temperature* under agitation
13. Rinse with ddH_2O (3x)
14. Suture coronary arteries closed to aid future cell seeding
15. Sterilize valves in 70% EtOH **overnight** at *room temperature* under agitation
    ---- STERILE CONDITIONS: INDIVIDUAL, STERILE, SPECIMEN-CUPS ----
16. Rinse with sterile PBS (3x)
17. Incubate valves in sterile ddH_2O **2 hours** at *room temperature* under agitation
18. Follow with tissue fixation

3.3.3 Tissue Fixation

Heart valve scaffolds were fixed to cross-link the proteins in the valve. They were treated overnight at room temperature under agitation with 0.075% penta-galloyl glucose (PGG) in 50mM dibasic sodium phosphate buffer in saline with 20% isopropanol, pH 5.5. The materials and methods follow.

Materials (for 500mL):

- Decellularized heart valves
- ddH_2O
- 70% EtOH
- Sterile 1x PBS
- Saline (3.6g NaCl in 400 mL ddH_2O)
- 0.075% PGG (Slowly add 400 mL phosphate buffer to 100mL PGG solution and run through 0.22 µM sterile filter system)
    - Phosphate Buffer - 50mM Na_2HPO_4 (2.84g Na_2HPO_4 in 400 mL saline, **pH: 5.5**)
    - 0.375g PGG dissolved in 100 mL isopropanol
    - 0.22 µM sterile filter system

Methods:

1. Rinse with sterile PBS (3x)
2. Incubate valves in sterile 0.075% PGG **overnight** at *room temperature* under agitation
1. Rinse with sterile PBS (3x)
2. Sterilize valves in 70% EtOH **20 minutes** at *room temperature* under agitation
3. Rinse with sterile PBS (3x)
4. Follow with cell seeding or store in sterile 0.02% NaN_3

3.3.4 Neutralization and Cellular Seeding

The valve scaffolds were incubated at least 3 hours at room temperature in sterile Delbecco's Modified Eagle Medium with 50% fetal bovine serum and 2% antibiotics/antimicotics to neutralize any phenolic groups from the PGG that may have been left after fixation. After aspirating the neutralization solution, the valves were stood upright in individual specimen cups and seeded with the appropriate number of cells in 200 µL of cell culture media for each cusp. Additional cell culture media was placed on the valves following mounting into the bioreactor.

3.3.5 Live/DEAD® staining

The LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Molecular Probes) is a two-color fluorescence assay used to stain cells on the surface of the cusps. Intracellular esterase activity distinguishes live cells via the enzymatic conversion of calcein AM to calcein, which intensely fluoresces green in live cells. The kit also uses EthD-1, which

enters cells with damaged membranes and fluoresces upon binding to nucleic acids, indicating dead cells upon its bright red fluorescence.

The assay was prepared as directed and placed on cusps in a six-well plate. After the incubation time in the dark, the samples were imaged with the fluorescence microscope (Invitrogen, 2005).

Materials (for 10mL):

- 1x PBS
- Six-well culture plates
- Live/DEAD® Viability/Cytotoxicity Assay Solution (Molecular Probes) (Vortex the EthD-1 and PBS, then add calcein and vortex)
    - 20 µL EthD-1
    - 5 µL 4mM calcein
    - 10mL 1x PBS

Methods:

1. Dissect valve leaflet from the valve
2. Rinse leaflet with PBS (1x)
3. Place leaflet in a well of the six-well culture plate
4. Add ~3mL stain to each well
5. Incubate in Live/DEAD® solution **20 minutes** at *37°C* in the dark
6. Image using FITC and Texas Red filters to examine the cells

3.3.6 MTS

MTS is the common name for the CellTiter 96® AQueous One Solution Cell Proliferation Assay. It is a colorimetric method used to determine the number of viable cells present in culture. The reagent combines a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] and an electron coupling reagent (phenazine ethosulfate; PES) to form a stable solution. The compound is added to the cells, where it is bioreduced into a colored formazen product by dehydrogenase enzymes in metabolically active cells. The number of living cells is directly proportional to the quantity of formazan product as measured by

the absorbance at 490 nm (Promega, 2007). For this assay, the reagent was added to the cell culture, incubated for at least one hour, and the absorbance read at 490nm with a 96-well plate reader.

3.3.7 Diff-Quick

Diff-Quick staining is a type of Romanowski stain based on a combination of eosin and methylene blue. It was originally designed to incorporate pink cytoplasmic staining with blue nuclear staining and fixation as a single step. For this assay, the leaflet was fixed in the Diff-Quick fixative for 30 seconds, and then rinsed with PBS. Following fixing, the tissue was stained in Diff-Quick solution II for 10 seconds, rinsed with PBS, counterstained with Diff-Quick solution I for 10 seconds, rinsed with PBS, and viewed under the light microscope.

3.3.8 Scanning Electron Microscopy

Scanning electron microscopy was used to image the cusp surface for a three-dimensional view of any cells present. After careful dissection from the valves, the cusps were placed and stored in Karnovsky's fixative (2.5% glutaraldehyde, 2% formaldehyde in 0.1 cacodylate, pH=7.4). Following dehydration with ethanol and coating with gold, the samples were imaged using the Hitachi S4800.

3.3.9 Hematoxylin and Eosin Sections

Hematoxylin & Eosin (H&E) staining is a popular staining method in histology. It uses the basic dye, hematoxylin, to color basophilic structures, such as those containing nucleic acids like the cell nucleus, a blue-purple hue. The acidic dye, eosin, colors

eosinophilic structures, such as cytoplasm and some extracellular matrix products, bright pink (Ross, Kaye et al., 2003).

Following fixation in 10% formalin, cusps were cut in half, embedded in paraffin wax, sectioned, and stained according to the following routine H&E staining method. Orientation of the sections was in the radial direction, crossing (vs. aligning with) the collagen bundles.

1. Xylene ---------- 10 dips
2. Xylene ---------- 5 min
3. 100% EtOH ---------- 10 dips
4. 100% EtOH ---------- 1 min
5. 95% EtOH ---------- 10 dips
6. 95% EtOH ---------- 1 min
7. Running Water ----------Till Clear
8. Distilled Water ---------- 1 min
9. Hematoxylin ---------- 5 min
10. Running Water ----------Till Clear
11. Clarifier ----------10-15 dips
12. Running Water ----------Till Clear
13. Bluing ---------- 1 min
14. Running Water ---------- 30 sec
15. 95% EtOH ---------- 10 dips
16. Eosin ---------- 45 sec
17. 95% EtOH ---------- 10 dips
18. 95% EtOH ---------- 10 dips
19. 100% EtOH ---------- 10 dips
20. 100% EtOH ---------- 10 dips
21. 100% EtOH ---------- 10 dips
22. Xylene ---------- 10 dips
23. Xylene ---------- 5 min

3.3.10 DAPI Sections

DAPI (4'-6-Diamidino-2-phenylindole) is a florescent stain that will pass through an intact cell membrane and bind strongly to dsDNA. Thus, it is used extensively in fluorescence microscopy to stain both live and fixed cells with a corresponding emission of bright blue fluorescence.(Invitrogen, 2006)

After performing routine H&E staining, DAPI staining helped validate the presence of nucleic acids that were difficult to distinguish. Each fixed and paraffin-embedded section was brought back to water according to the following steps and stained directly on the slide.

1. Xylene ---------- 10 dips
2. Xylene ---------- 5 min
3. 100% EtOH ---------- 10 dips
4. 100% EtOH ---------- 1 min
5. 95% EtOH ---------- 10 dips
6. 95% EtOH ---------- 1 min
7. Running Water ----------Till Clear

3.4 Heart Valve Experimentation

The experimental usefulness of the bioreactor was tested using a living valve; decellularized, fixed, and seeded valves; and a tri-layered valve made from pericardium and scaffolding. In all, fifteen experiments were performed. These consisted of the following four types; one set of results will be shown for each type.

3.4.1 Valve 1: Living Valve – 7 Days

Valve preparation

Valves were collected from Snow Creek Meat Processing, Seneca, SC and cleaned as described in section 3.3.1. Modifications to the above protocol consist of cleaning the valves in the sterile hood instead of on the lab bench.

Bioreactor conditions

Following cleaning, the living valve was mounted in the bioreactor in DMEM with 10% fetal bovine serum and 1% antibiotics/antimycotics. That valve was then tested at 60 bpm and 60 mL of air per stroke for seven days inside the incubator at 37°C and 5% CO_2. A static control valve was kept in a specimen cup in the incubator, next to the bioreactor.

Analysis

After dissection, the cusps of the valves were cut away from the valve wall and the MTS, Live/DEAD and Diff-Quick assays were performed as listed in Table 2.

Table 2: Experimental conditions & assays for living valve experiment (3.4.1).

ID		Cusp	Number of Days	Conditioning specifications				Assay Performed
				beats per minute	stroke volume (mL)	°C	% CO_2	
Valve 1	1-150-Living	1 a	7	60	15	37	5	Live/Dead
		1 b						DiffQuick
		2 a						Live/Dead
		2 b						MTS
		3 a						Live/Dead
		3 b						MTS
Valve 2	1-150-Static	1 a	7	0	0	37	5	Live/Dead
		1 b						DiffQuick
		2 a						Live/Dead
		2 b						MTS
		3 a						Live/Dead
		3 b						MTS

3.4.2 Valve 2: Endothelial Cell Seeded Valve – 21 Days

Valve preparation

Eight valves were collected from Snow Creek Meat Processing, Seneca, SC and cleaned as described in section 3.3.1. Following decellularization (3.3.2 – Modifications include step 2: Incubate valves in ddH_2O **overnight** at *room temperature* under agitation) and tissue cross-linking (3.3.3), the valves were stored in 0.02% NaN_3. Four valves (see Table 3) were used for this experiment as selected by visual opening and closing between step 14 and 15 in section 3.3.2: Tissue Decellularization. These valves included a time zero day, non cell-seeded valve (T0DC), a time zero day, cell-seeded valve (T0C), a time 21 day static control (T21S), and a time 21 day dynamic test valve (T21D).

Cellular specifications

After seven days of storage, the four selected valves were rinsed and stored in sterile saline for two days at 4℃ to remove any residual NaN_3. This was followed with neutralization and cell seeding (3.3.4). 1E5 porcine aortic endothelial cells at passage 6 were seeded per cusp. MCDB with 10% fetal bovine serum and 2% antibiotics/antimycotics without cells was used as a control for valve T0DC.

Bioreactor conditions

The test valve was mounted in the bioreactor and progressive adaptation over the course of 20 hours was used to bring the pumping conditions to 60 bpm and 10mL per stroke. DMEM with 10% fetal bovine serum and 2% antibiotics/antimycotics was used and changed every seven days for all samples. Of note is that after the first seven days, the culture media in the bioreactor began to appear cloudy. The cell culture media in the system was changed as soon as possible after noticing this. Cloudiness did not return for the remainder of the experiment. Static controls as listed in Table 3 were placed in specimen cups in the incubator next to the bioreactor at 37°C and 5% CO_2.

Analysis

After one night in the incubator and dissection of cusps from the valve wall, the cusps of valves T0C and T0DC were analyzed according to the assays listed in Table 3. After 21 days, the cusps of valves T21D and T21S were analyzed according to the assays listed in Table 3.

Table 3: Experimental conditions & assays for endothelial cell experiment (3.4.2).

ID		Cusp	Cells	Number of Days	Conditioning specifications				Assay Performed		
					beats per minute	stroke volume (mL)	°C	% CO_2			
Valve 1	2-083-T0C	1 a	1E5	over-night	0	0	37	5	SEM		
		1 b									
		2 a	1E5						DNA (not performed)		
		2 b							Protein (not performed)		
		3 a	1E5						Live/Dead	H&E	DAPI
		3 b									
Valve 2	2-083-T0DC	1 a	0	over-night	0	0	37	5	SEM		
		1 b									
		2 a	0						DNA (not performed)		
		2 b							Protein (not performed)		
		3 a	0						Live/Dead	H&E	DAPI
		3 b									
Valve 3	2-083-T21D	1 a	1E5	21	60	10	37	5	SEM		
		1 b									
		2 a	1E5						DNA (not performed)		
		2 b							Protein (not performed)		
		3 a	1E5						Live/Dead	H&E	DAPI
		3 b									
Valve 4	2-083-T21S	1 a	1E5	21	0	0	37	5	SEM		
		1 b									
		2 a	1E5						DNA (not performed)		
		2 b							Protein (not performed)		
		3 a	1E5						Live/Dead	H&E	DAPI
		3 b									

3.4.3 Valve 3: Adipose-derived Stem Cell Seeded Valve – 21 Days

This experiment was performed in collaboration with Dr. Aggie Simionescu and Christopher Albers.

Valve preparation

Valves were collected from Snow Creek Meat Processing, Seneca, SC and cleaned as described in section 3.3.1. Decellularization (3.3.2) and tissue cross-linking (3.3.3) followed. Conditions for the two valves used for this experiment can be seen in Table 4.

Cellular specifications

After neutralization the valves were seeded (3.3.4) with rat adipose derived stem cells. 5E4 cells/cm^2 were seeded in each cusp.

Bioreactor conditions

The test valve was mounted in the bioreactor in DMEM with 10% fetal bovine serum and 1% antibiotics/antimycotics and progressive adaptation was used to bring the pumping conditions to 60 bpm and 10mL per stroke. A static control was placed in a specimen cup in the incubator next to the bioreactor at 37°C and 5% CO_2. Media was changed every seven days for all samples.

Analysis

After 21 days and dissection of cusps from valve walls, the cusps of valves T21D and T21S were analyzed according to the assays listed in Table 4.

Table 4: Experimental conditions & assays for stem cell experiment (3.4.3).

ID		Cusp	Number of Days	Conditioning specifications				Assay Performed	
				beats per minute	stroke volume (mL)	°C	% CO_2		
Valve 1	2-118-T721D	1 a	7	60	10	37	5	Live/DEAD	
		1 b						H&E	DAPI
		2 a						Live/DEAD	
		2 b						H&E	DAPI
		3 a						Live/DEAD	
		3 b						H&E	DAPI
Valve 2	2-118-T21S	1 a	7	0	0	37	5	Live/DEAD	
		1 b						H&E	DAPI
		2 a						Live/DEAD	
		2 b						H&E	DAPI
		3 a						Live/DEAD	
		3 b						H&E	DAPI

3.4.4 Valve 4: Tri-layered Tissue Engineered Heart Valve – 8 Days

This experiment was performed in collaboration with Dr. Dan Simionescu and Mary E. “Betsy” Tedder.

Valve preparation

Porcine pericardium and porcine pulmonary artery from 2+ year pigs were received from Animal Technologies, Inc., Tyler, Tx, and cleaned of all tissue and extraneous fat/loose connective tissue over wet ice. Following decellularization (3.3.2), the tissue was subjected to elastase treatment (10 u/mL). Figure 8 shows the process of forming the valve from these scaffolds. To form the tri-layered valves, silicon molds and counter molds were made from aortic valves. Those counter molds were then used to shape the collagen pericardium scaffolding into aortic roots and leaflets. After cross-linking (3.3.3) of the new valve, the original leaflets of the valves were removed and the

scaffolds were then sutured to the original aortic root from where it was created, forming the ventricularis and fibrosa layers of an aortic tricuspid valve.

Cellular specifications

Following a rinsing, the valve and external pulmonary scaffold were neutralized (3.3.4) and each pulmonary artery scaffold was seeded with 5E4 human bone marrow-derived stem cells. Figure 7 shows the pulmonary scaffold after insertion between the two layers of pericardium scaffolding, which were later glued together to form the pocket. After one night in the bioreactor, a leaflet that was impeding the opening and closing of the other two was dissected and its spongiosa layer was placed in a six-well plate as a static control.

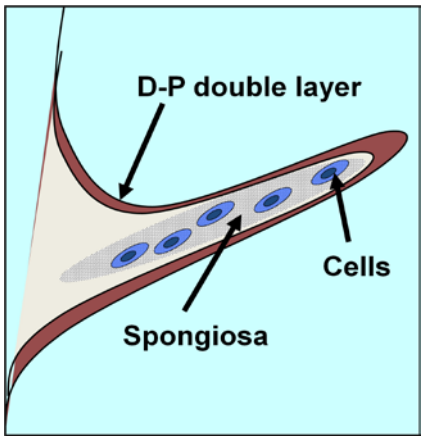


Figure 7: Cross sectional view of the tri-layered leaflet.
(Courtesy of Dr. Dan Simionescu)

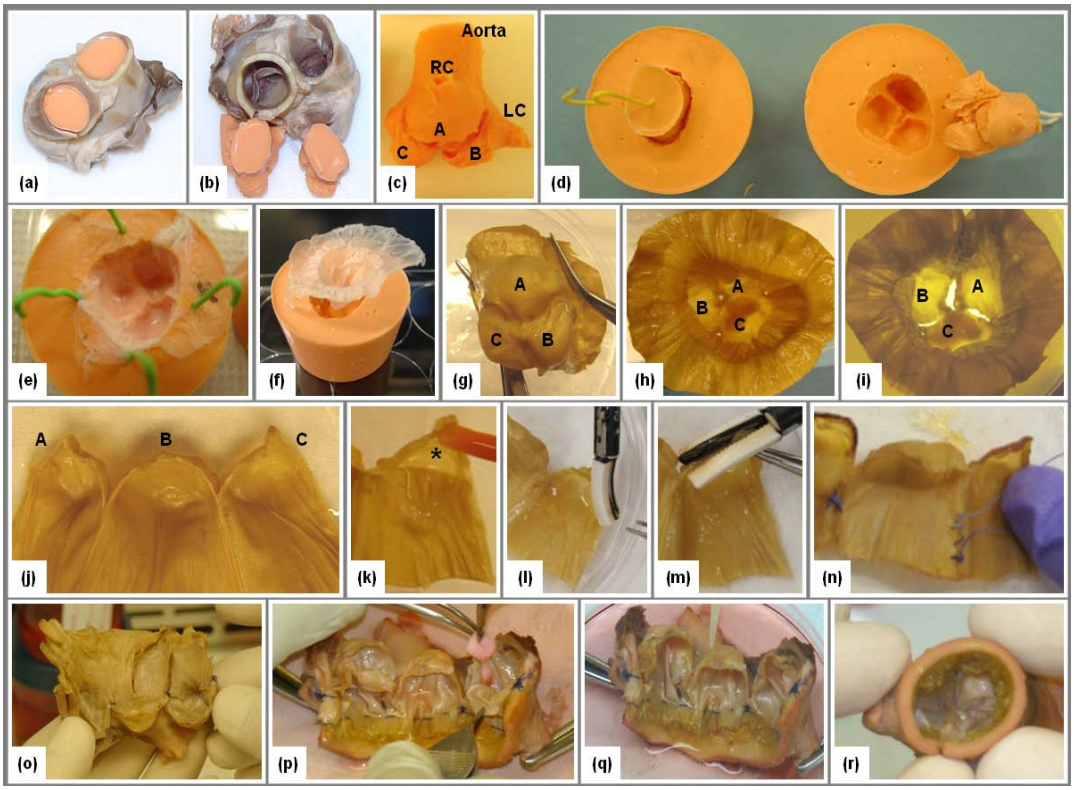


Figure 8: Formation of the tissue engineered heart valve. (a-d) silicon molds and counter-molds made from aortic valves. **(e-i)** layered collagen scaffolds shaped as aortic roots. **(j)** Aortic root constructs were cut open to show internal structures. Final construct was prepared by gluing layers at the cusp levels **(k)**, welding free edges **(m)** and suturing above the sinuses **(n)**. Gluing and welding were done here only for demo purposes (see next steps). The construct was then sutured to the original aortic root (from which the silicon mold was created) maintaining the proper orientation **(o)**; cell-seeded spongiosa was then placed between the two fibrous layers **(p)**, the cusp edges sealed with glue **(q)** and the aortic root closed and rinsed for implantation into the heart valve bioreactor **(r)**. (Data and photos courtesy of Dr. Dan Simionescu and Mary E. "Betsy" Tedder)

Bioreactor conditions

The valve was mounted into the bioreactor in DMEM with 10% fetal bovine serum and 1% antibiotics/antimycotics and progressive adaptation over a time period of three days was used to bring the pumping conditions to 60 bpm, 33% inspiration time, and approximately 18 mL per stroke. Media in the bioreactor was not changed, but media in the static control was changed every three days. The static control was placed in the incubator next to the bioreactor at 37°C and 5% CO_2.

Analysis

After a test period of eight days, the spongiosa layers of the valves were analyzed according to the assays listed in Table 5.

Table 5: Experimental conditions & assays for tri-layered tissue engineered heart valve (3.4.4).

Valve	Cusp	Section	Number of Days	Conditioning specifications				Assay Performed
				beats per minute	stroke volume (mL)	°C	% CO_2	
TEHV	1	a	8	60	18	37	5	Live/Dead
		b						DAPI
		c						DAPI + Vimentin
TEHV	2	a	8	60	18	37	5	Live/Dead
		b						DAPI
		c						DAPI + Vimentin
TEHV	3	a	8	0	0	37	5	Live/Dead
		b						DAPI
		c						DAPI + Vimentin

CHAPTER 4: RESULTS

4.1 Bioreactor Design

A pneumatic-driven conditioning system (Figure 9), was developed that consists of a three-chambered heart valve bioreactor (1), a webcam for viewing (2), a pressurized compliance tank (3), a reservoir tank (4), one-way valves (5), pressure-retaining valves (6), pressure transducers (not shown), an air filter for gas exchange (7), and a pump (Siemens 900E) to power the cyclic fluid flow (8). The acrylic bioreactor can be sterilized using conventional methods and accommodates all clinically relevant sizes of stented or stentless biological, mechanical or tissue engineered substitutes. Other features of the bioreactor include an unobstructed observation area for the camera, a modular design allowing easy replacement of cell culture media, and multiple ports for media sampling.

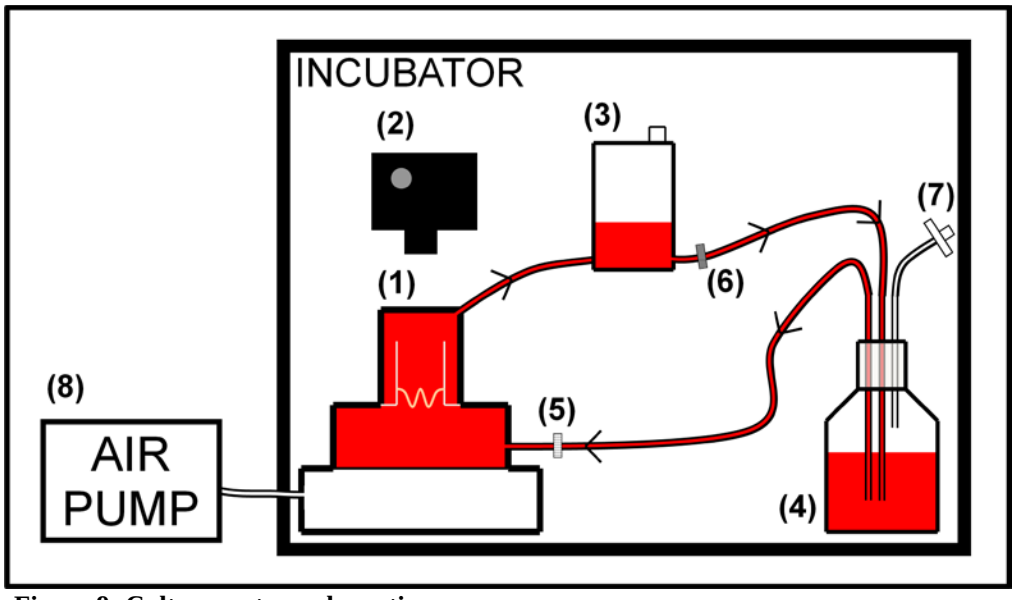


Figure 9: Culture system schematic

The developed bioreactor seen in Figure 10 is composed of three transparent compartments. Made of acrylic plastic, it is 6 inches in diameter, approximately 8.5 inches tall, and completely transparent. The three parts of the bioreactor are held together by stainless steel screws.

The air chamber (A) is connected to the external pump and is the only chamber not filled with culture medium. It is separated from the pumping chamber (B) by clear silicone rubber. Through the power of the external air pump, this membrane bulges into the pumping chamber and pushes the residing media through the heart valve (C) into the aortic chamber (D). Once completed, the pump releases pressure in the air chamber, allowing the membrane to fall down and draw culture medium in through the one way valves from the reservoir tank to fill the pumping chamber in preparation for the next cycle. Figure 11 demonstrates this pumping action.

During the pumping phase, the curvature of the pumping chamber and angle of media inlets ensures consistent distribution of culture medium throughout the chamber. Once through the valve, the medium enters the aortic chamber then flows into the compliance chamber. The clear, flat top of the aortic chamber facilitates the viewing of the functioning of the heart valve. Both the aortic and pumping chambers have multiple ports for easy access of pressure transducers, media in/outlets, or other probes.

The special removable valve holder in the design is able to adapt to valves of all sizes and types. The holder is mounted to the inferior side of the aortic chamber with stainless steel screws. With the addition of a sealing cover, the aortic chamber has the additional benefit of creating a sterile chamber that can be used to transport the valve.

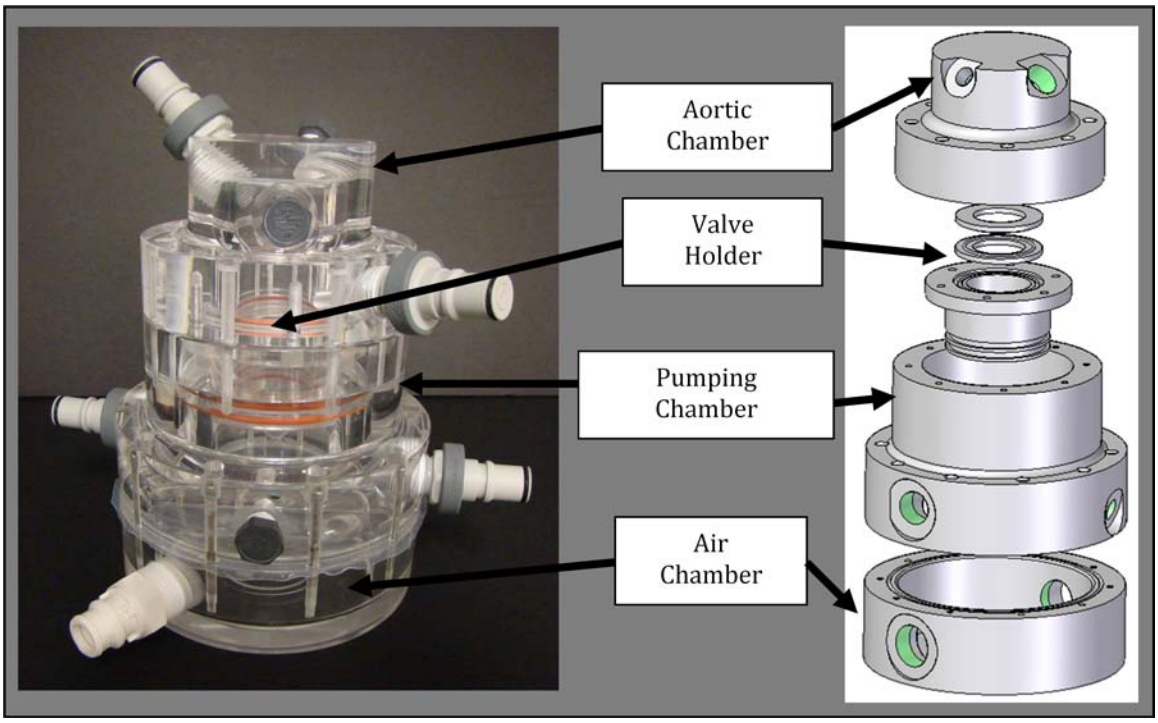


Figure 10: Picture and computer aided drafting representation of assembled bioreactor.

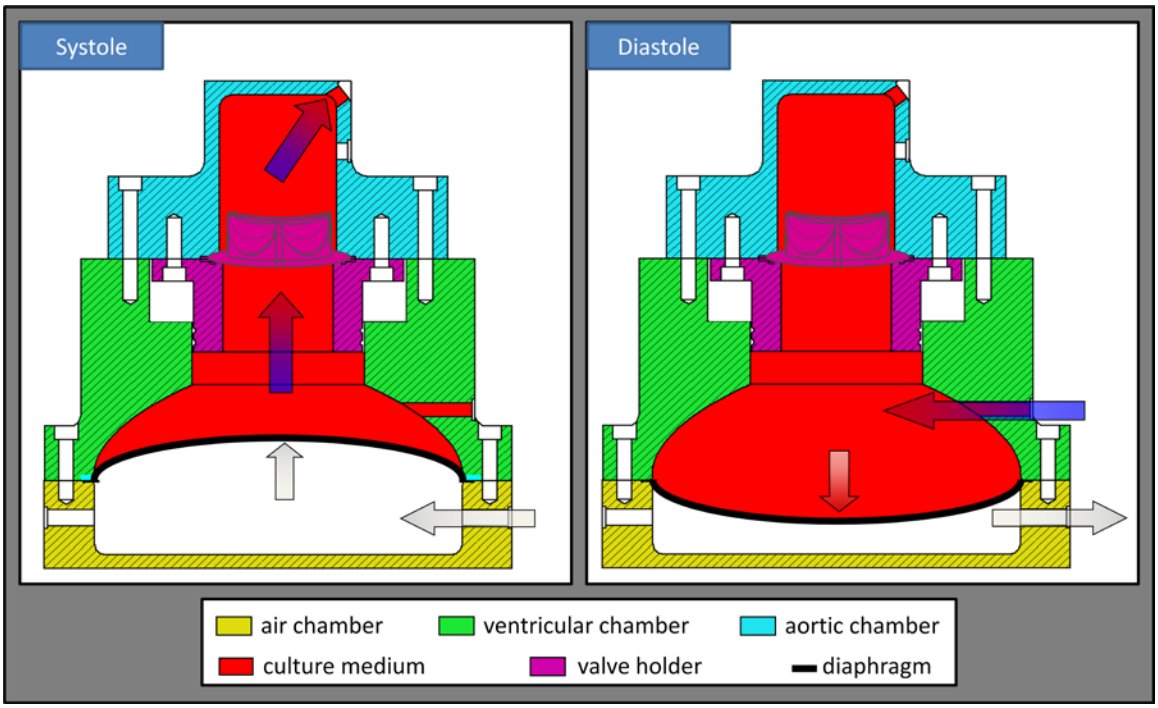


Figure 11: Cross-sectional view of the bioreactor demonstrating the pumping actions.

4.2 Bioreactor Capabilities Testing Using a Bioprosthetic Heart Valve

The desired environmental conditions of the bioreactor as a pump included 1) aortic systolic/diastolic pressures reaching at least 120/80 mmHg, 2) flow rates reaching 5000 mL/min, 3) stroke rate exceeding 60 beats per minute, 4) oxygen and carbon dioxide levels appropriate to support the growth of the cells and tissues, and 5) repeatability of these conditions.

Testing the conditioning system design revealed that most of these conditions were met. Sections 4.2.1 and 4.2.2 show results related to the desired pressures and flows, respectively.

4.2.1 Pressure

Section 3.2.1 describes the setup for pressurizing the system. Under these conditions, values of pressure were recorded under no pressurization, after an initial pressurization of the top reservoir using compressed air, after a second pressurization of the top reservoir, and after all pressure was released from the system in both the aortic chamber and ventricular chamber of the bioreactor. Figure 12 illustrates pressures experienced in this experiment. Figure 13 details the non-pressurized section and Figure 14 shows the sustained pressurized section. Figure 12 and Figure 13 reveal the effects of taking readings through the DAQ at 180 Hz. Only three data points were plotted per second, creating a “low resolution” pressure profile.

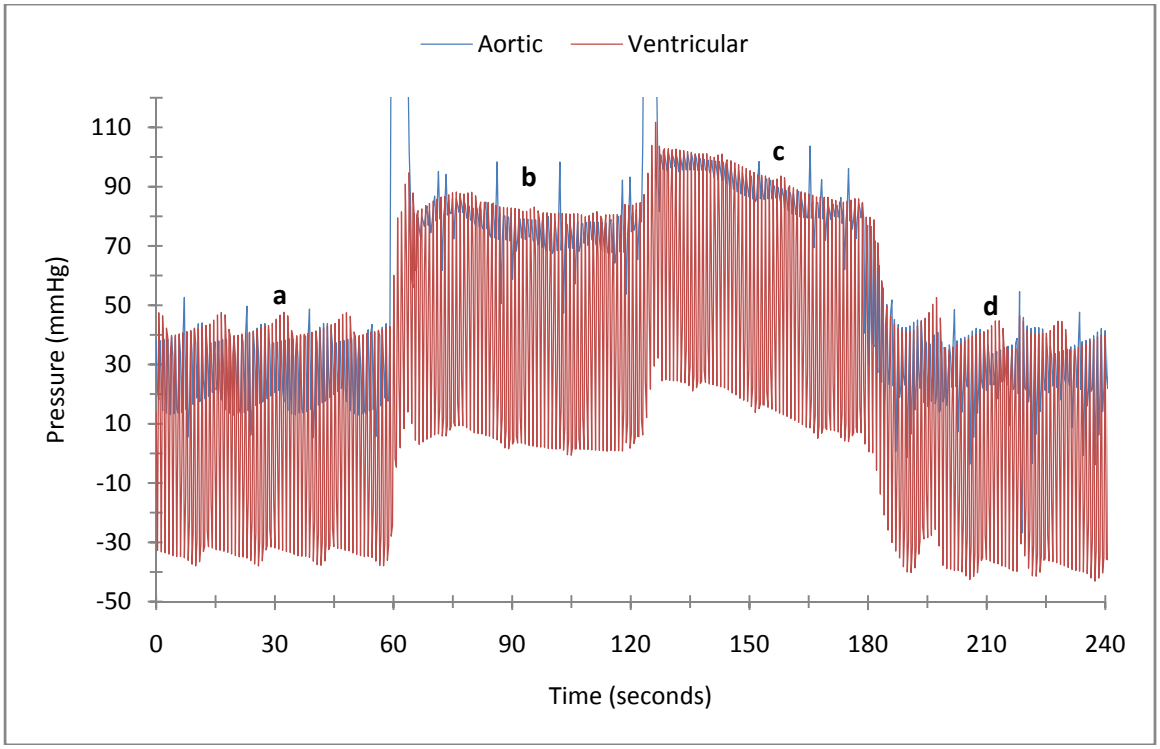


Figure 12: Chamber pressures. a) no added pressure b) initial pressurization c) additional pressurization d) release of all pressure

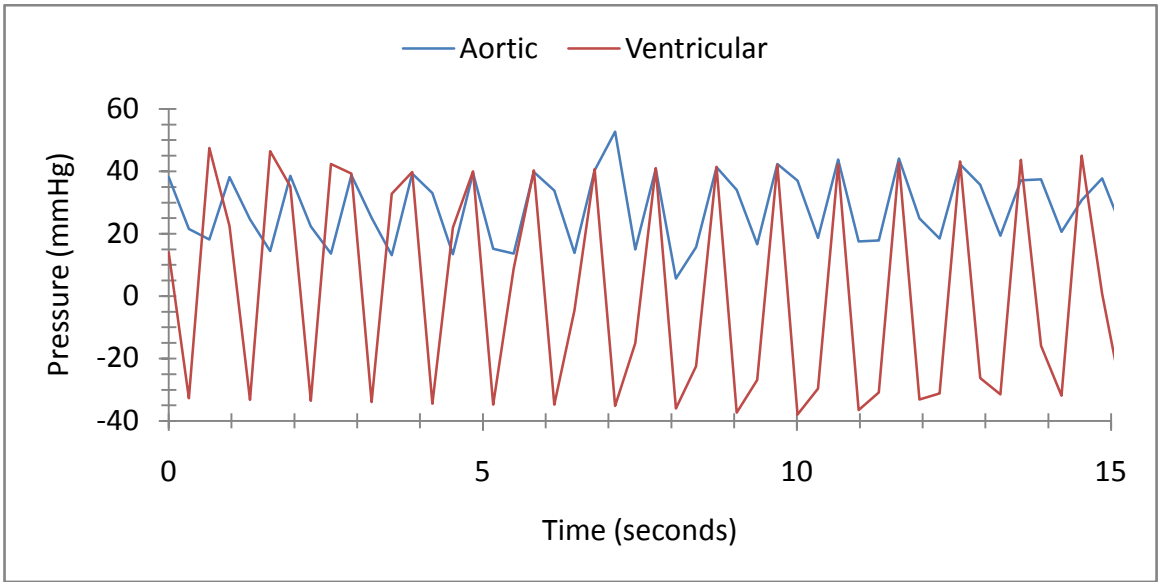


Figure 13: Chamber pressures without external application of pressure

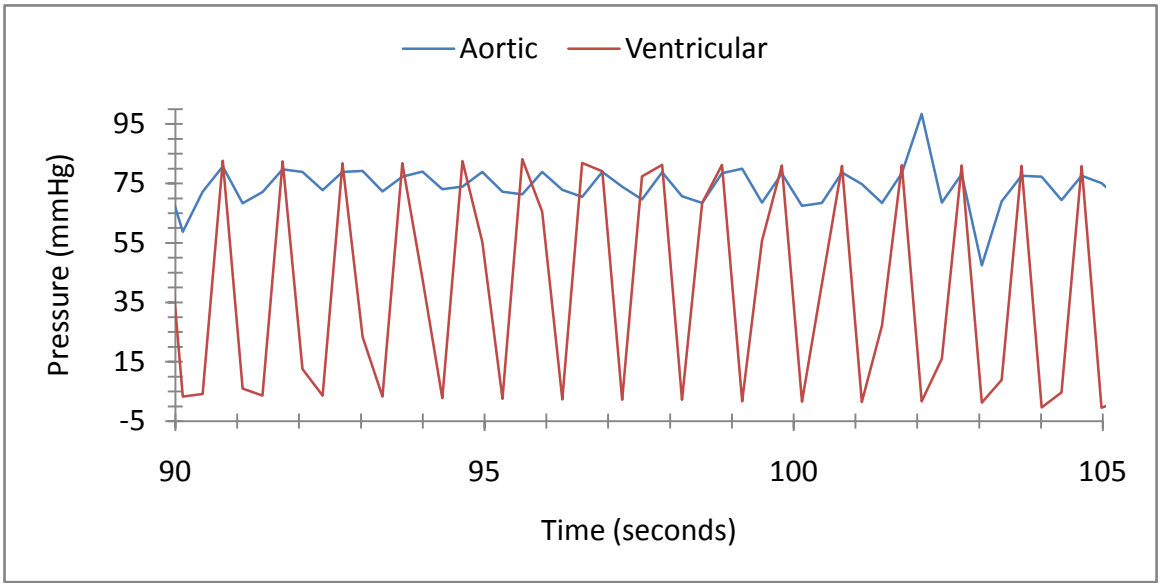


Figure 14: Chamber pressures with external application of pressure

4.2.2 Flow

As section 3.2.2 described, fluid flow through the system was measured at increasing, incremental values. Table 6 shows the resulting stroke volumes at 60 bpm for as the value of inspiration tidal volume of air (mL) set for the ventilator is increased. As a result, the total volume of fluid flow per minute ranges from 284 mL/min to 1336 mL/min. Of note is that for inspiration tidal volume air settings of 7.5 and 10 mL, the BHV was only slightly opening. Figure 15 shows a graphical representation of the same data, that can be used to find the ventilator settings necessary to achieve a desired stroke rate. Figure 16 shows the same for the total fluid flow per minute.

Table 6: Stroke volumes at 60 beats per minute

Inspiration Tidal Volume Air (mL)	Stroke Volume (mL)
7.5*	4.7
10*	6.2
20	8.6
50	14.3
80	17.5
100	18.7
130	22.3

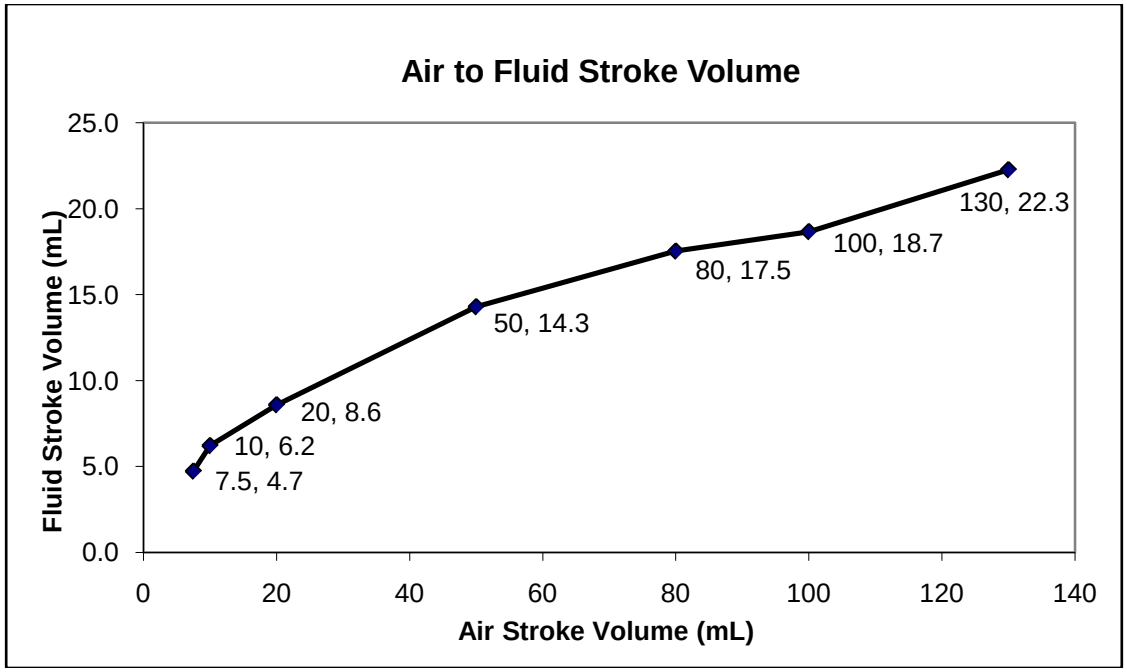


Figure 15: Stroke volume for multiple settings of the ventilator. Use this graph to determine how to set the the air stroke volume on the ventilator for a desired fluid stroke volume through the valve.

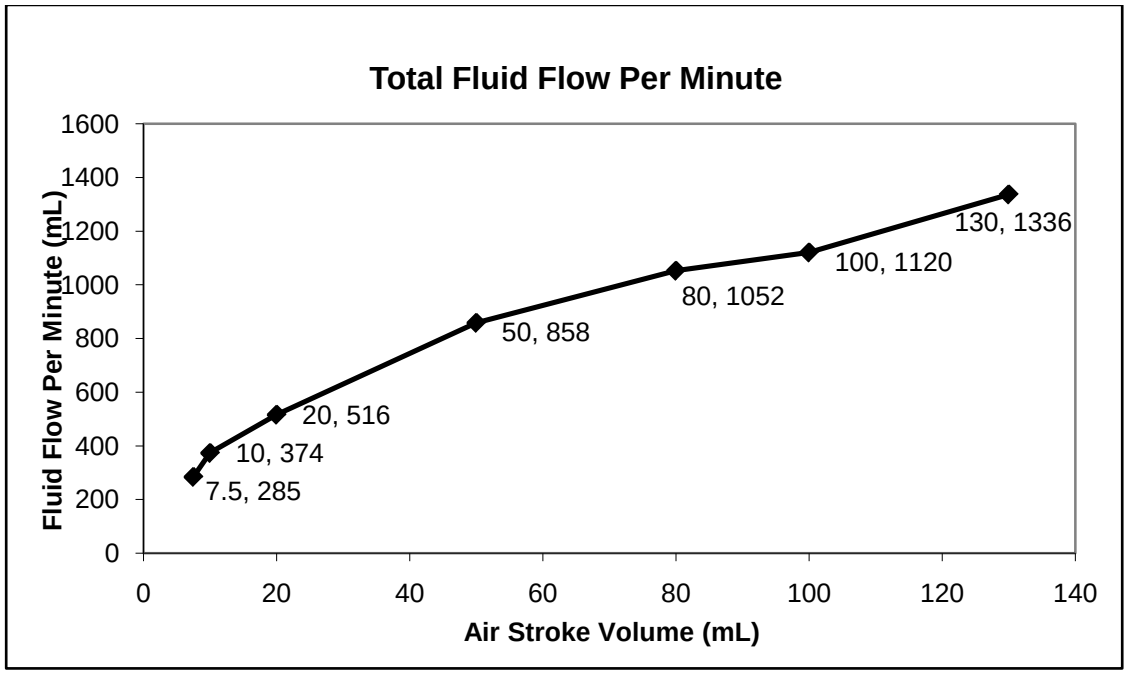
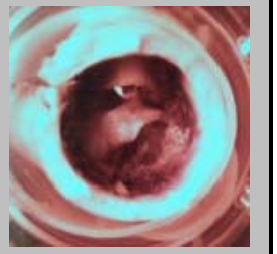
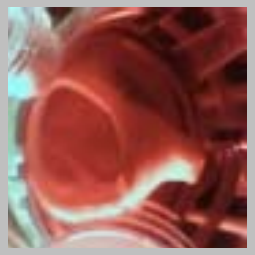
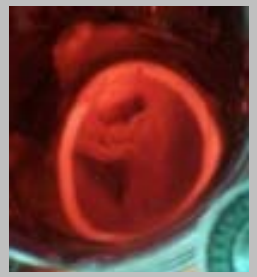
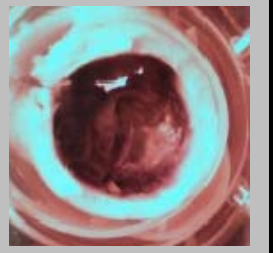


Figure 16: Total fluid flow through the valve per minute.

4.3 Heart Valve Experimentation

Fifteen experiments tested four types of valves. The first was a living valve that was mounted within hours of harvesting. The second and third valves were decellularized, fixed, and seeded with either porcine aortic endothelial cells or rat adipose-derived stem cells, respectively. The fourth valve was made of decellularized, fixed, valve-shaped fibrous sheets encasing a spongy collagen scaffold seeded with human bone marrow-derived stem cells. Figure 17 shows macroscopic images of these valves with a summary of the experimental conditions for each. The bioreactor successfully maintained an environment conducive to cell survival and differentiation. It provided the desired factors for the desired amount of time. Figure 17 shows an overview of each valve and detailed results for each valve type are below.

Valve Name	Test Valve – BHV	Valve 1 – Living	Valve 2 – ECs	Valve 3 – ASCs	Valve 4 - TEHV
Open					
Closed					
Decellularization	Yes	No	Yes	Yes	Yes
Fixation	Glutaraldehyde	No	0.075% PGG	0.075% PGG	0.075% PGG
Neutralization	None	None	50% Serum	50% Serum	50% Serum
Cell Type	None	Native	Porcine Aortic Endothelial Cells	Rad Adipose-derived Stem Cells	Human Bone Marrow-derived Stem Cells
Seeding (cells/cm^2)	0	0	100,000	50,000	50,000
Progressive Adaptation	No	No	Yes	Yes	Yes
Stroke Volume	20	20	10	10	18
Days in Bioreactor	-	7	21	21	8

4.3.1 Valve 1: Living Valve – 7 Days

Visual analysis of the Diff-Quick staining revealed that the cells were less dense in the dynamic cusp than in the static cusp. Live/DEAD imaging revealed similar numbers of live cells between the two groups. However, a slightly higher number of dead cells were present in the dynamic cusp than in the static cusp. Figure 18 shows representative images of the Diff-Quick and Live/DEAD assays.

Table 7 shows the MTS assay supporting these findings in both the static and dynamic cusps. The increase in the cell solution's optical density (OD) after the incubation period in both the static and dynamic cusps signified the presence of living, metabolically active cells, but corresponding to the visual analysis of the Live/DEAD and Diff-Quick assays, the OD of the dynamic cusps was 15% lower than that of the static cusps.

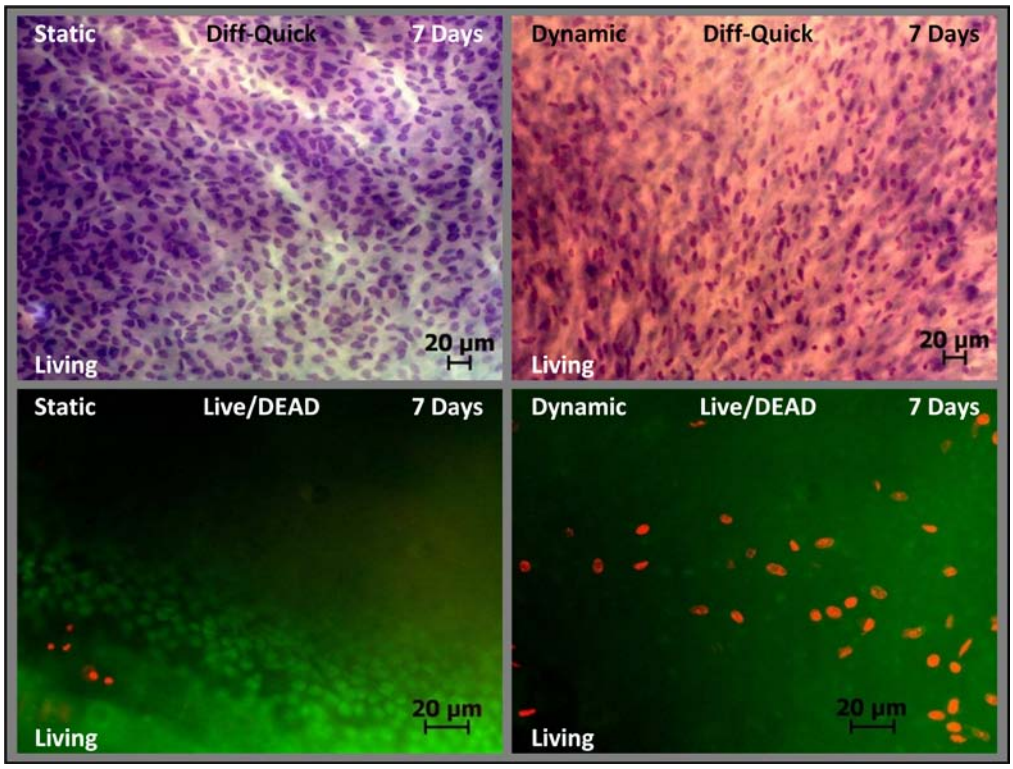


Figure 18: Results of the living valve experiment, 7 days.
Diff-Quick, nucleus = blue, cytoplasm = red/pink; Live/DEAD, live = green, dead = red.

Table 7: Optical density of culture solution via MTS assay.

	Optical Density		Wet Weight		Mean OD per mg Wet	Standard Deviation	OD per Avg Cusp Weight = 50mg
	Cusp 1	Cusp 2	Cusp 1	Cusp 2			
T7-Static	2.88	2.13	71.04	50.27	0.0414	0.00122	2.07
T7-Dynamic	1.50	1.44	40.58	42.19	0.0355	0.00190	1.77

4.3.2 Valve 2: Endothelial Cell Seeded Valve – 21 Days

Figure 20 and Figure 22 provide results from this experiment at time = 0 days. Figure 21 and Figure 23 reveal the results at time = 21 days. Histological sections show successful initial decellularization in all samples. Cells were successfully seeded and cells remained present on all appropriate groups for the entire experiment. While some cellular alignment is in all groups, there is notably more in the valve under dynamic conditions than in any other valve. SEM imaging revealed that most cells were located near the base of the cusp rather than near the free edge of the cusp. SEM imaging identified significant morphological, alignment, density, and locational differences.

Inspecting the solution of Live/DEAD stain used for the time = 21 days revealed many cells present in that solution after transferring the cusp. Figure 19 shows this solution and the cells that detached during manipulative handling.

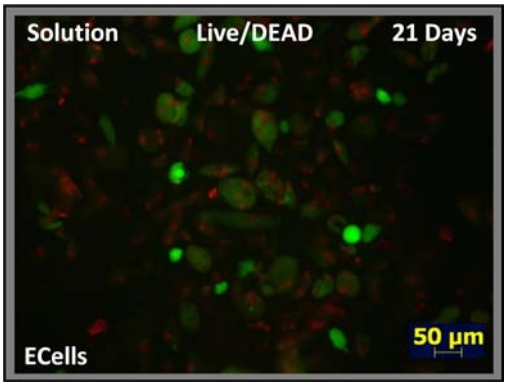


Figure 19: Live/DEAD staining solution.
Live/DEAD, live = green, dead = red.

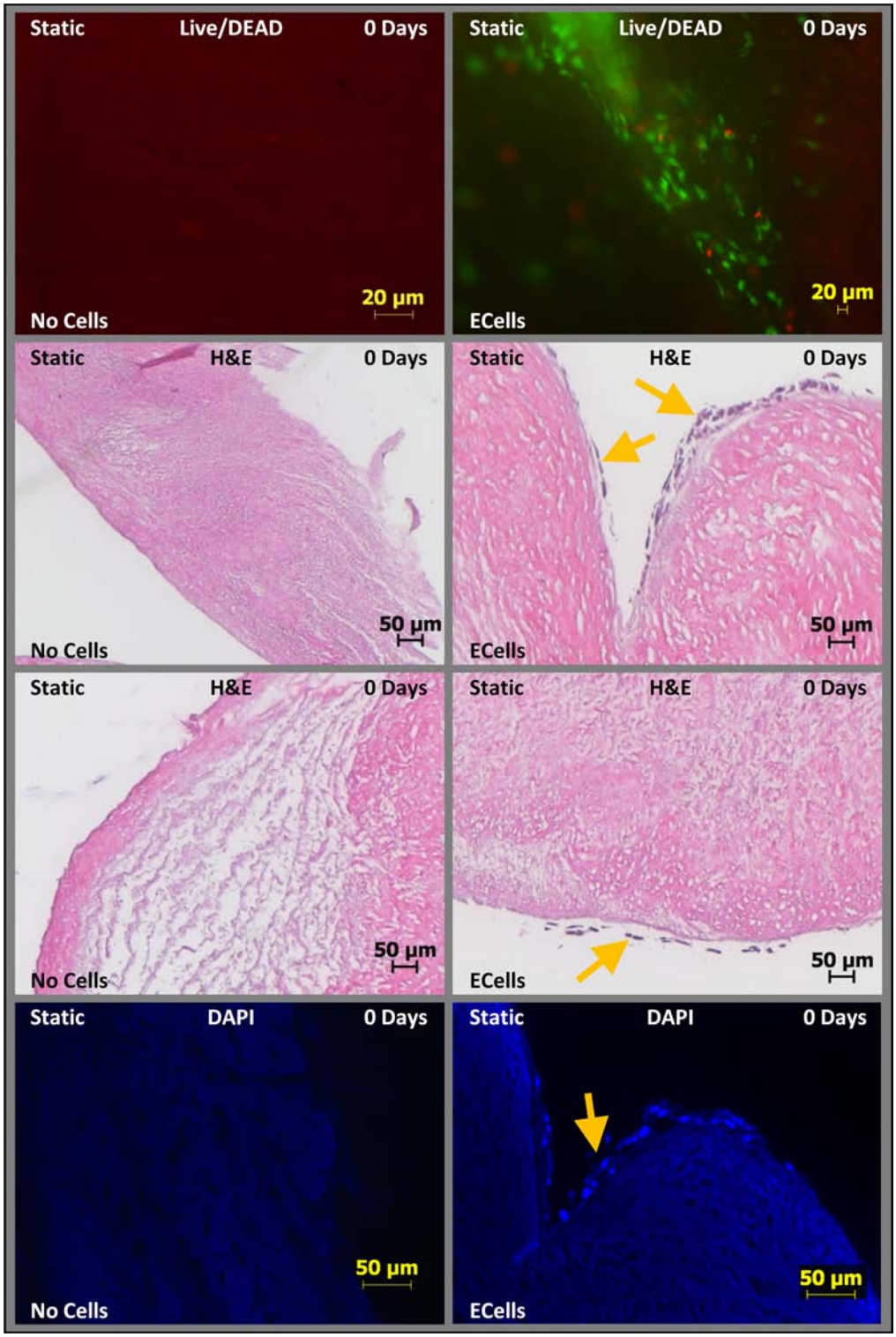


Figure 20: Results of the endothelial cell experiment, 0 days.
Live/DEAD, live = green, dead = red.; DAPI, nucleus = blue.
For ease of viewing, digitally altered images of DAPI staining can be found in Appendix B
(DAPI staining & photos courtesy of Mary E. "Betsy" Tedder)

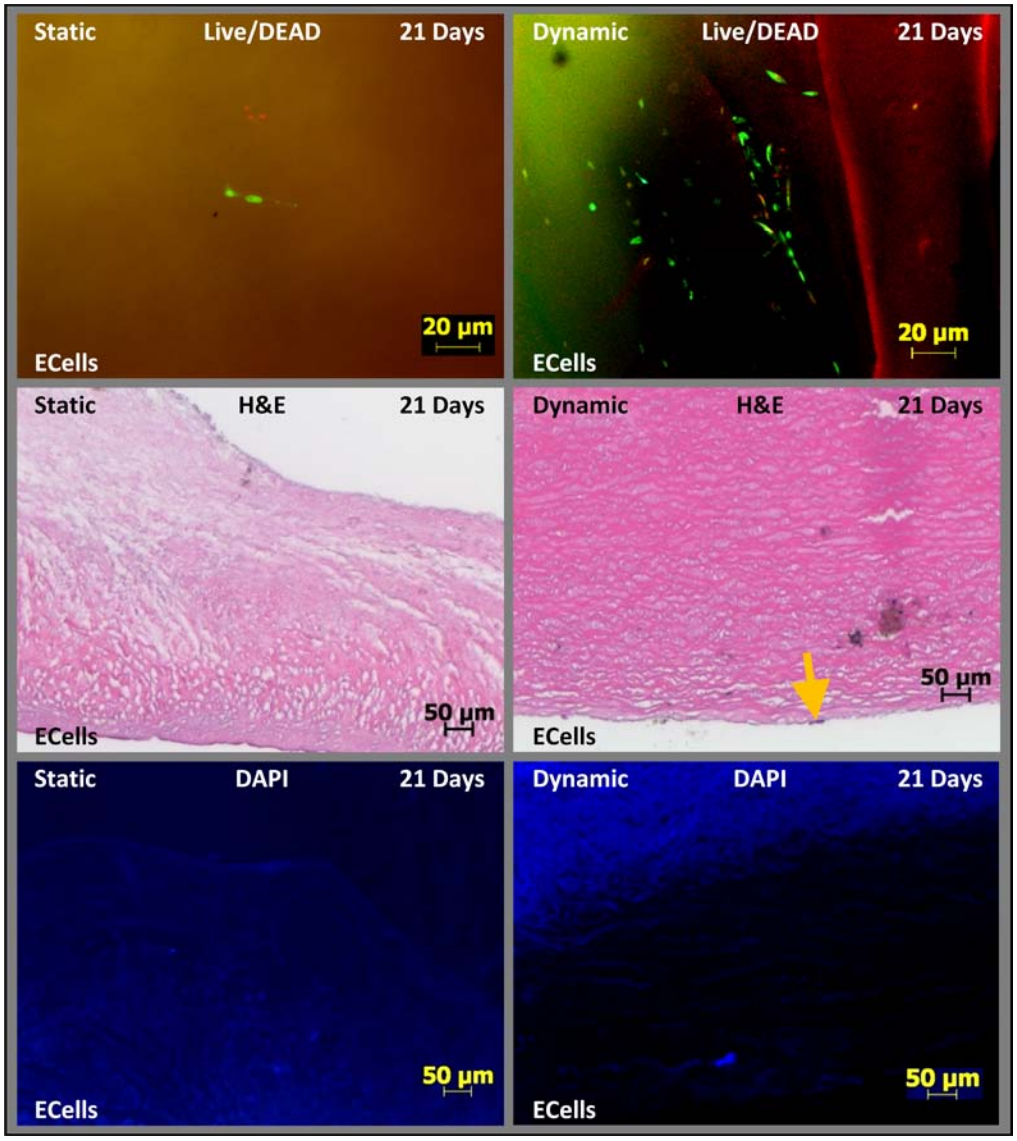


Figure 21: Results of the endothelial cell experiment, 21 days.
Live/DEAD, live = green, dead = red.; DAPI, nucleus = blue.
For ease of viewing, digitally altered images of DAPI staining can be found in Appendix B
(DAPI staining & photos courtesy of Mary E. "Betsy" Tedder)

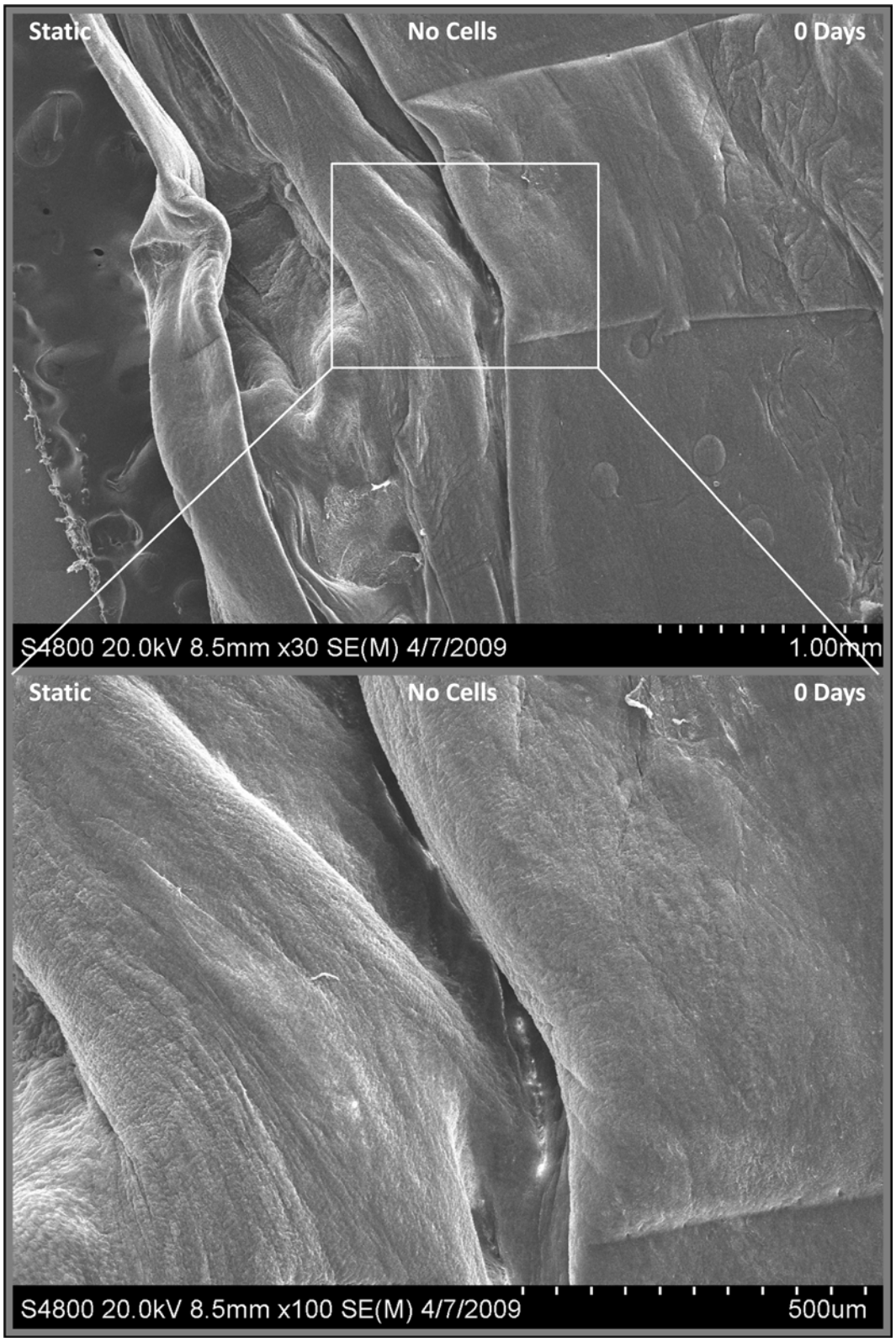


Figure 22: SEM images from the endothelial cell experiment, 0 days, no cells, static.

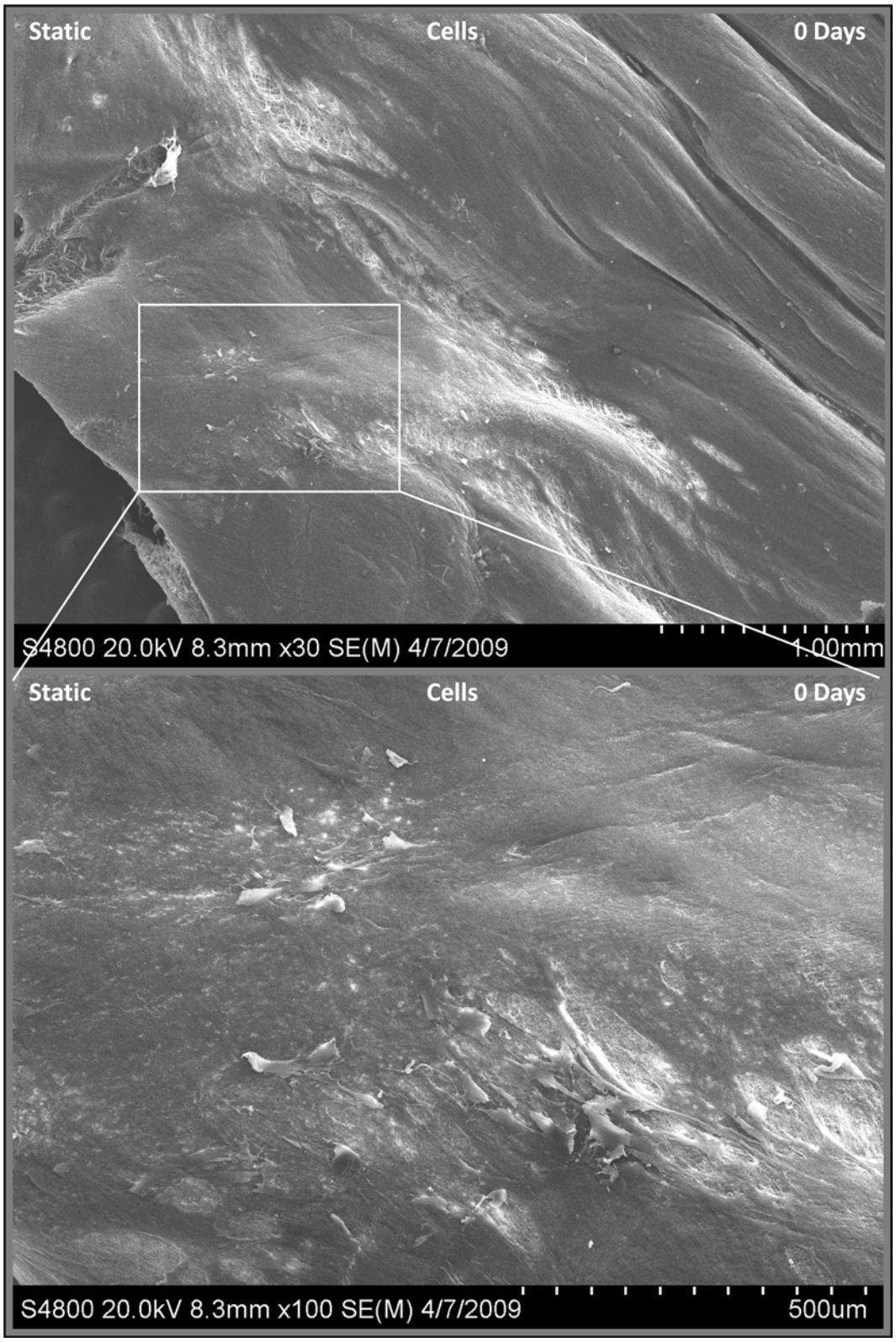


Figure 23: SEM images from the endothelial cell experiment, 0 days, cells, static.

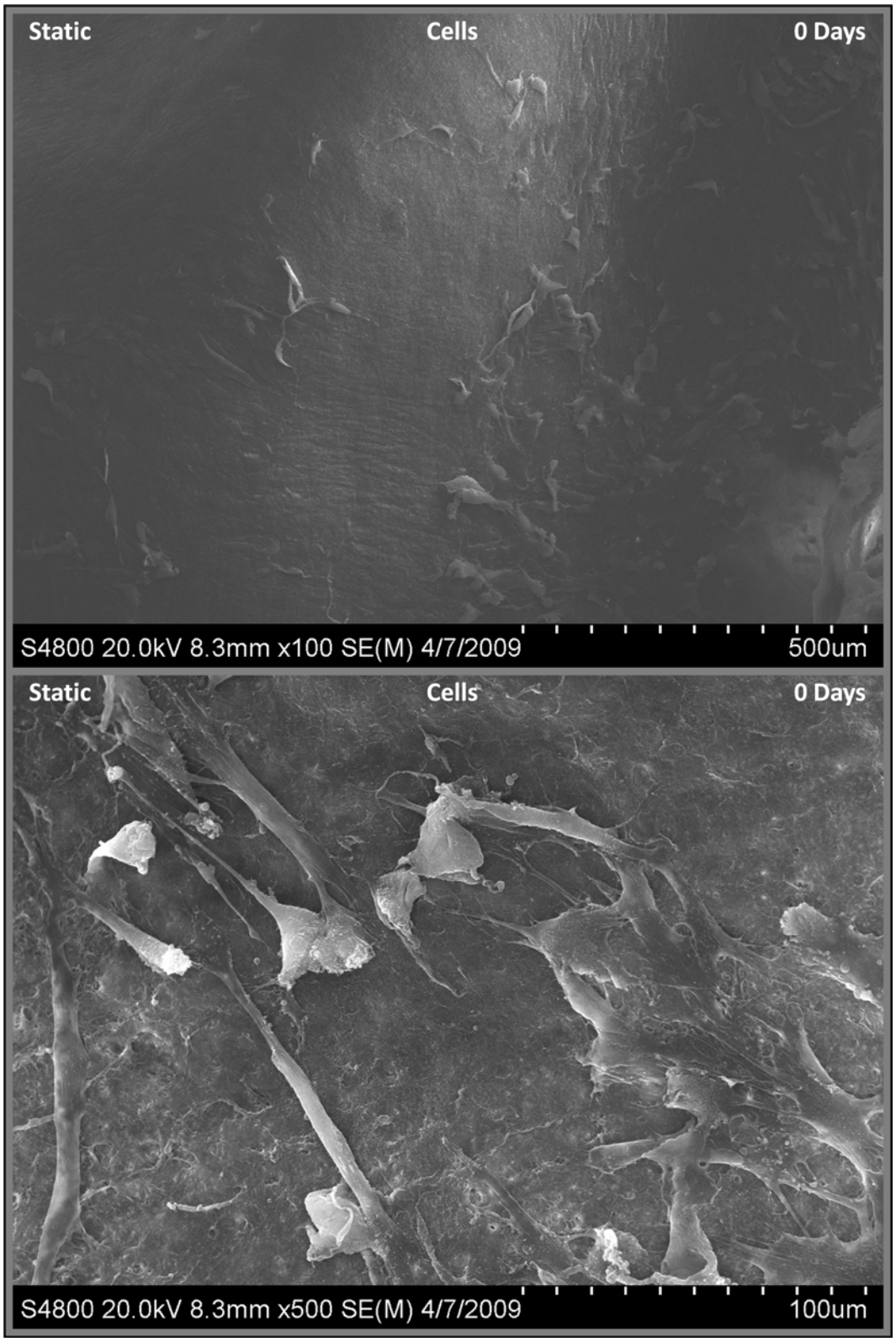


Figure 24: SEM images from the endothelial cell experiment, 0 days, cells, static.

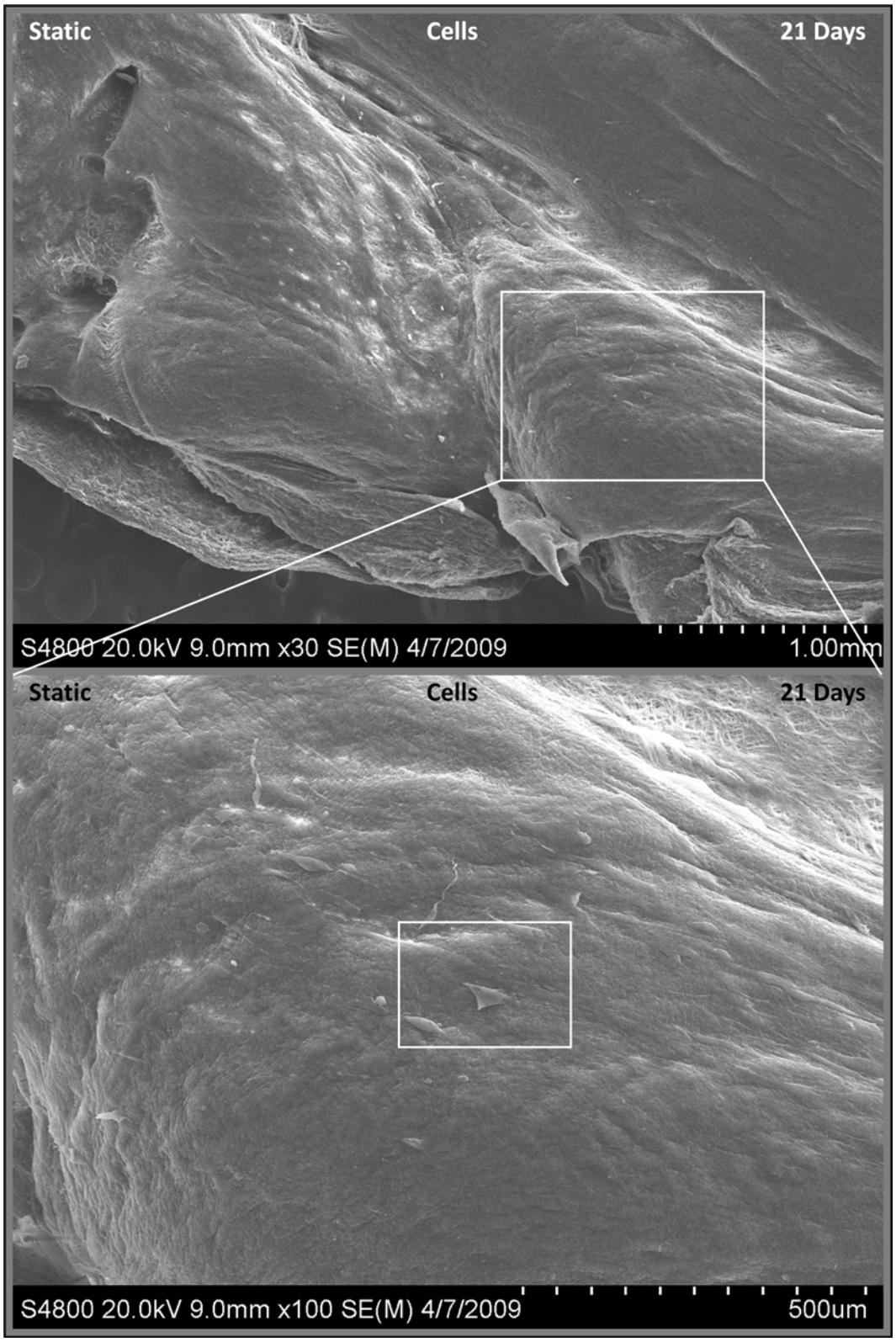


Figure 25: SEM images from the endothelial cell experiment, 21 days, cells, static.

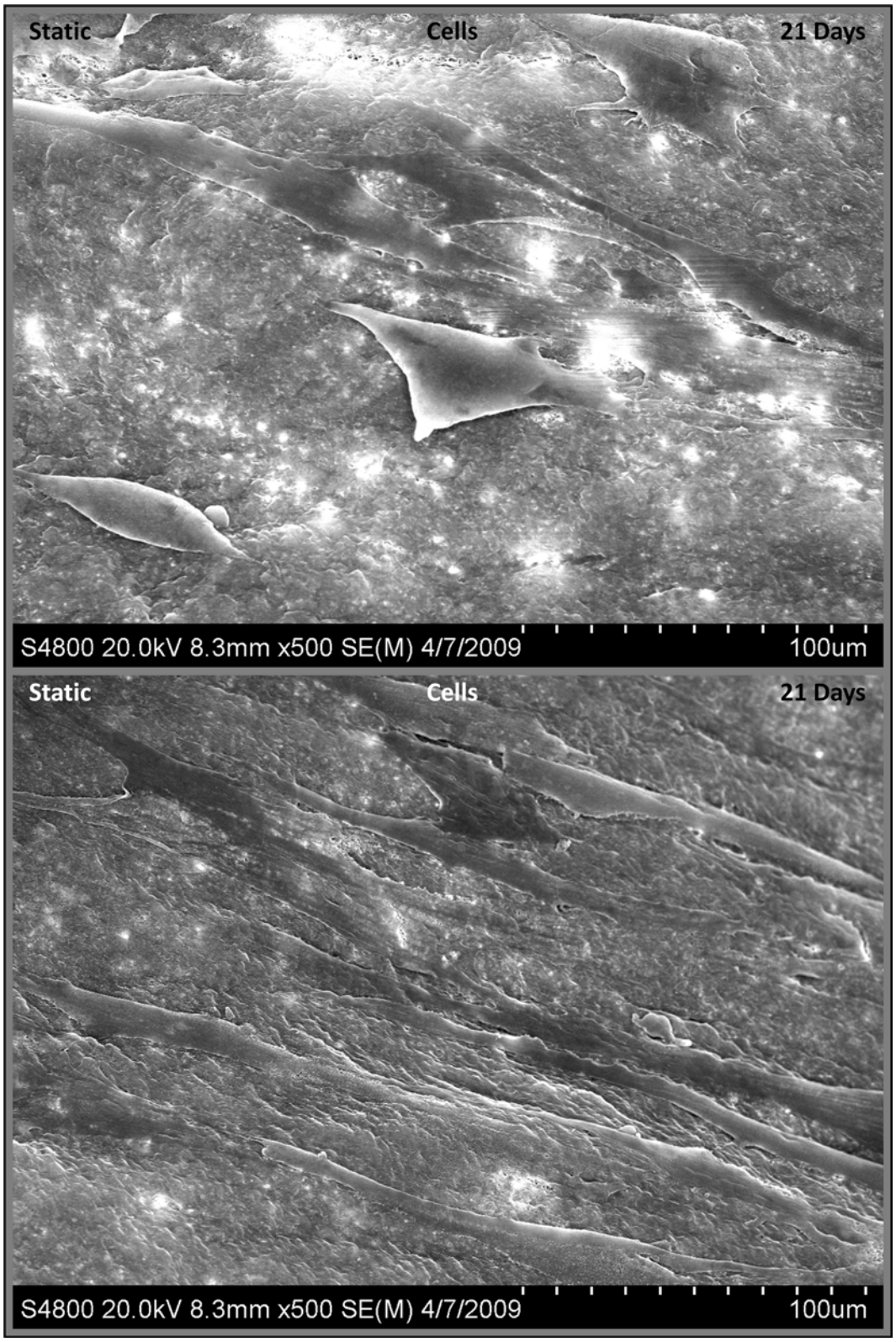


Figure 26: SEM images from the endothelial cell experiment, 21 days, cells, static.

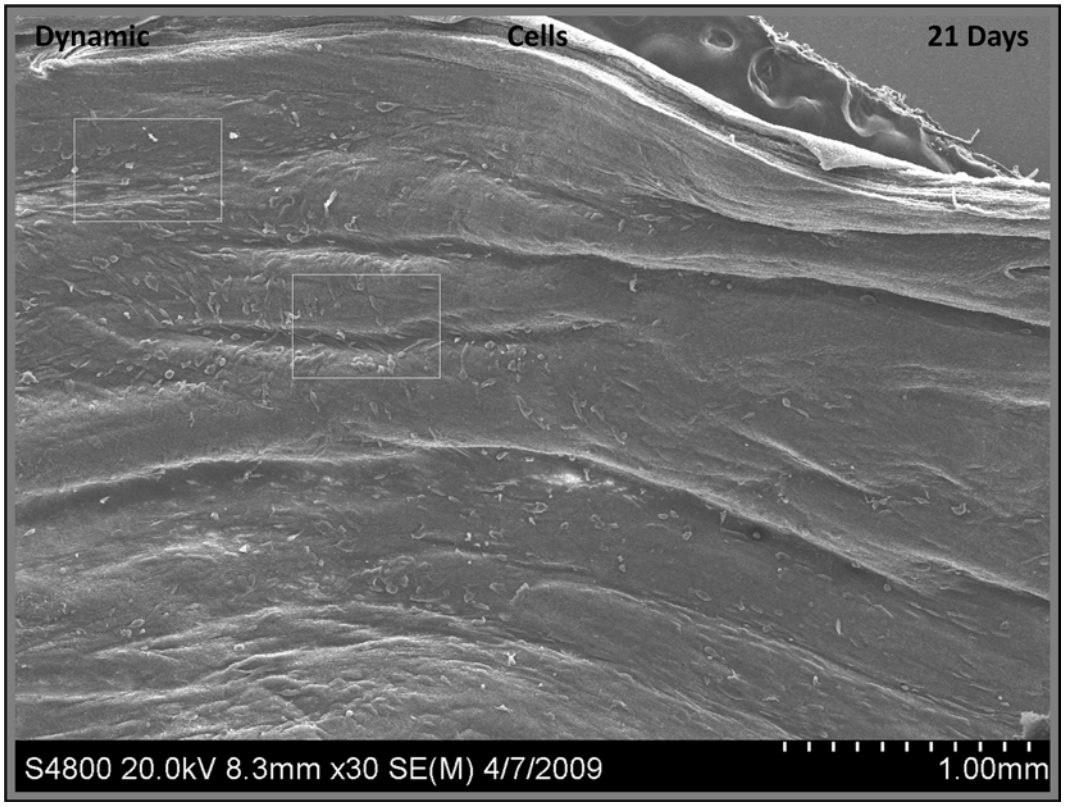


Figure 27: SEM images from the endothelial cell experiment, 21 days, cells, dynamic.

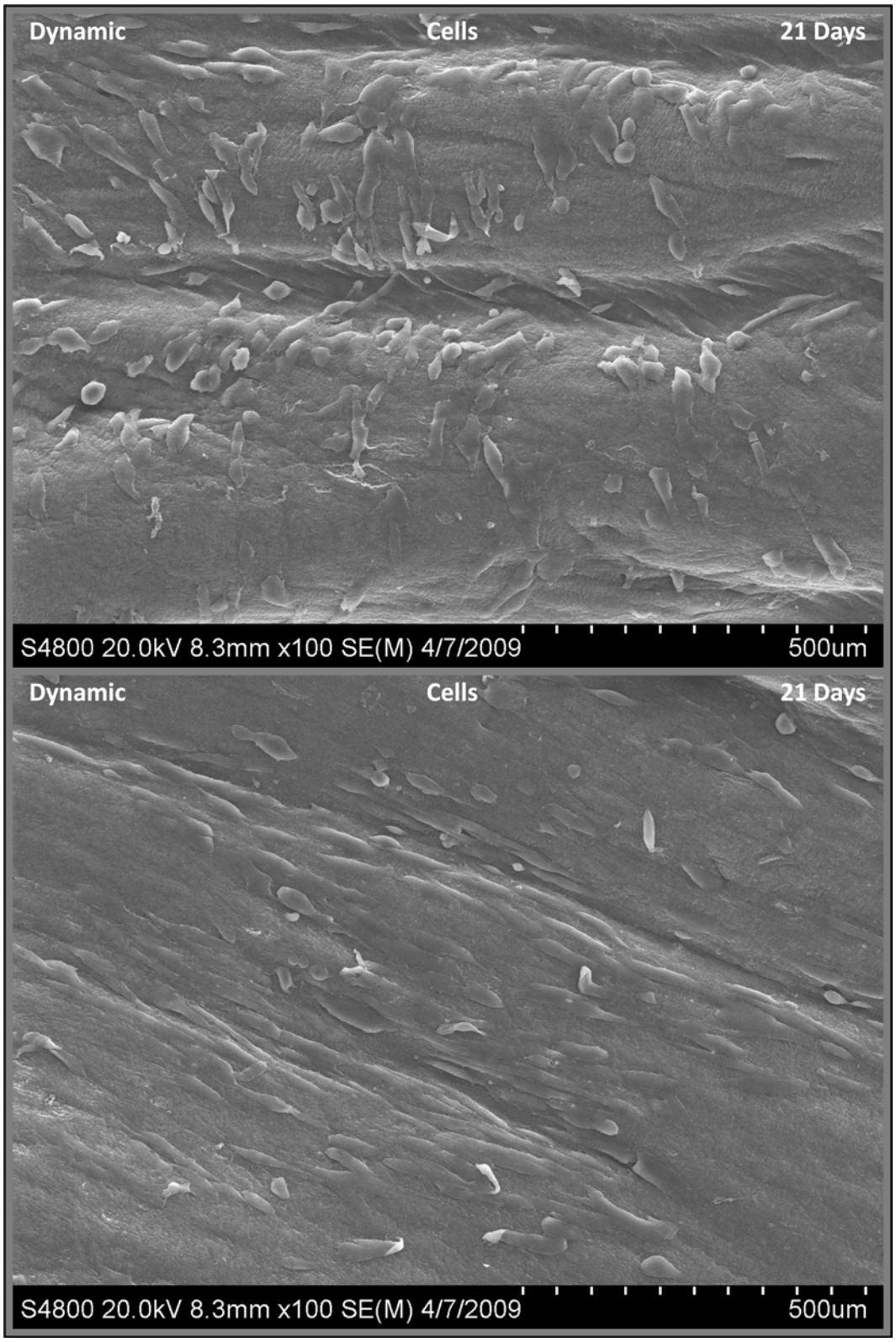


Figure 28: SEM images from the endothelial cell experiment, 21 days, cells, dynamic.

4.3.3 Valve 3: Adipose-derived Stem Cell Seeded Valve – 21 Days

Figure 29 reveals histological sections show successful decellularization of the cusps in this experiment. The yellow arrows notes the presence of seeded cells at time = 21 days. DAPI imaging confirmed the presence of cells at time = 21 days in the H&E sections.

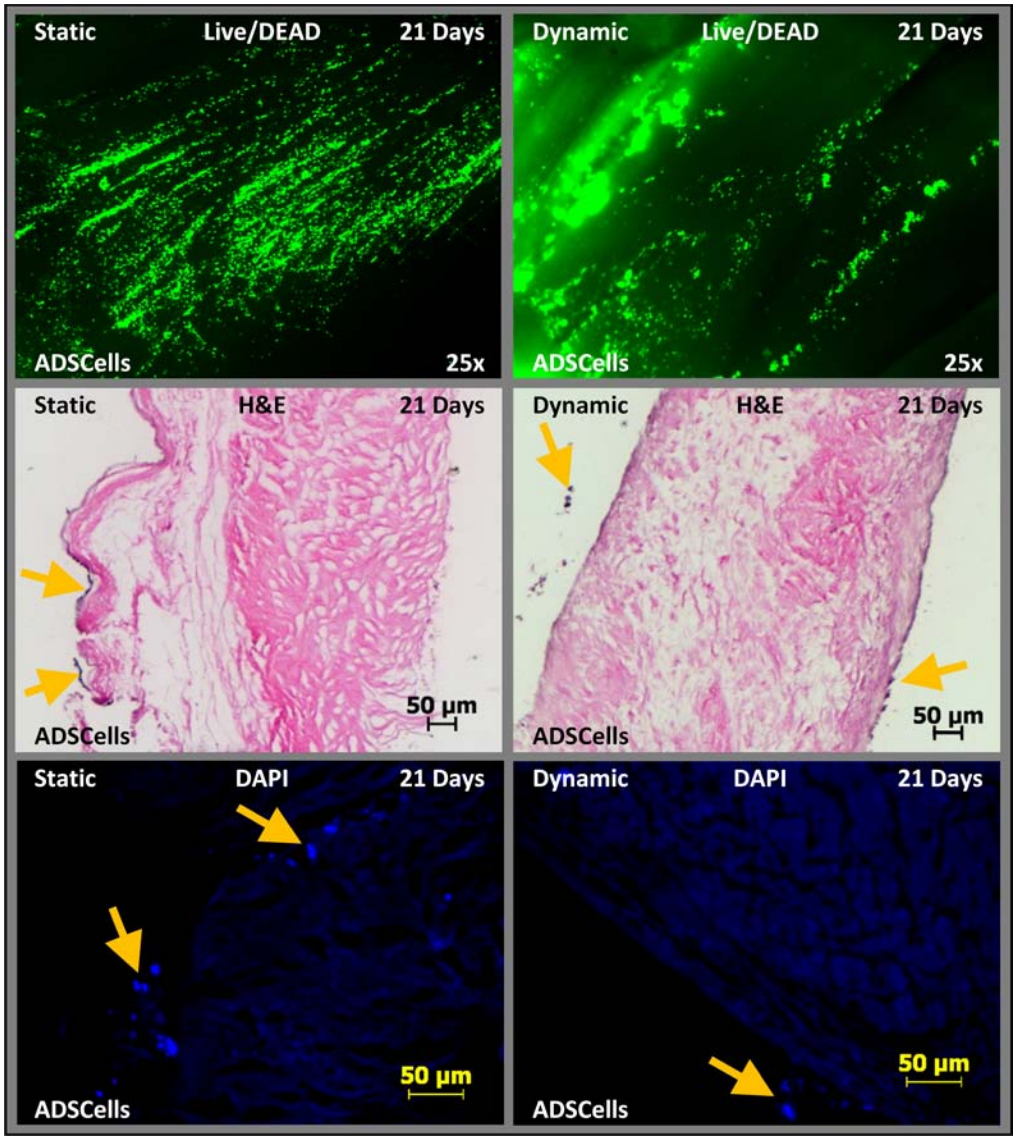


Figure 29: Results of the adipose-derived stem cell experiment, 21 days.
Live/DEAD, live = green; DAPI, nucleus = blue.
For ease of viewing, digitally altered images of DAPI staining can be found in Appendix B
Live/DEAD staining & photos courtesy of Dr. Aggie Simionescu and Christopher Albers)

4.3.4 Valve 4: Tri-layered Tissue Engineered Heart Valve – 8 Days

Figure 31 shows that human bone marrow-derived cells survived on the spongiosa layer of the tissue engineered heart valve in static and dynamic conditions for eight days. Cells in the dynamic conditions of the bioreactor were more elongated and stained more heavily for vimentin than those under static conditions. For comparison, Figure 30 includes images of porcine valvular interstitial cells stained for vimentin (red) and smooth muscle cell actin (red).

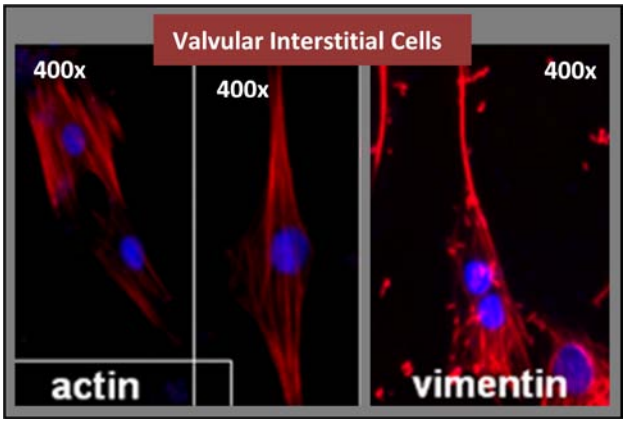


Figure 30: Actin vs. vimentin in valvular insterstitial cells for comparison.
actin = red, nucleus = blue, vimentin = red.
(Staining & photos courtesy of Dr. Dan Simionescu and Mary E. "Betsy" Tedder)

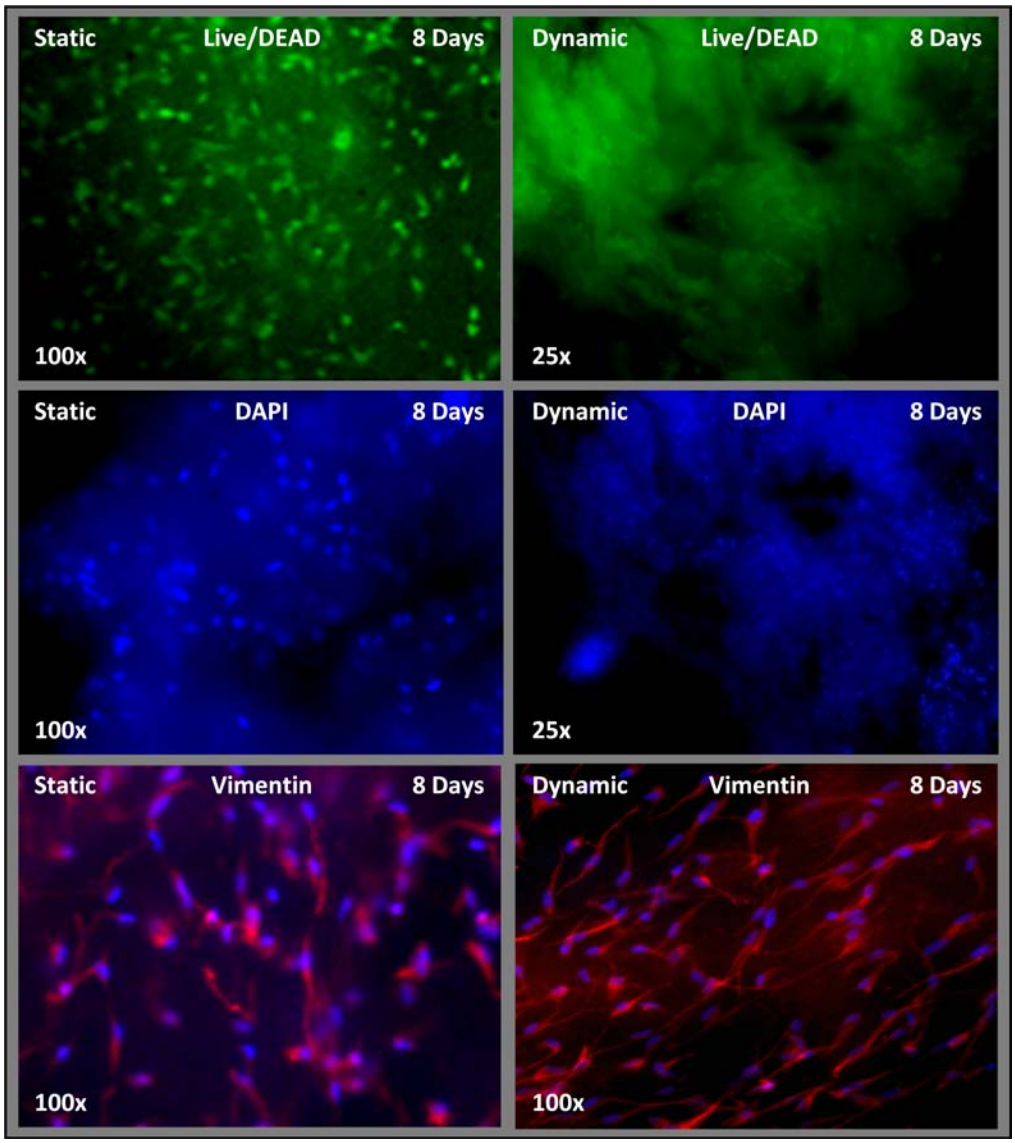


Figure 31: Results of the tissue engineered heart valve experiment, 8 days.
Live/DEAD, live = green; DAPI, nucleus = blue; Vimentin, nucleus = blue, vimentin = red. (Staining & photos courtesy of Dr. Dan Simionescu and Mary E. "Betsy" Tedder)

CHAPTER 5: ANALYSIS AND DISCUSSION

5.1 Bioreactor Design

The final product demonstrates that the objectives and wants of the customer were routinely reviewed during the design process to ensure that they were followed. The valve successfully opened and closed in every experiment, the system enabled pressures to be easily altered, and flow was adequate to ensure proper nutrient and waste transport. Throughout the project, the bioreactor was consistent, reproducible, non-toxic, and maintained the visibility of the heart valve using a webcam, which broadcasted the playback on the internet.

Unlike other heart valve bioreactors in the literature, the Clemson heart valve bioreactor is able to test all clinically relevant sizes of stented or stentless biological, mechanical, or tissue engineered valve substitutes. It's variable mounting method can adjust for variant thicknesses of the base, rigidities of the aortic root, and even amounts of material that would normally be used for mounting. Throughout the fifteen pilot tests, each variance in valves was seen and mounted at least once.

The greatest weakness of the Clemson bioreactor its ability to maintain a sterile environment. The large number of pieces, time it takes to assemble, and frequent media changes all contribute to the risk of contamination. Eventually, as we learned how to properly handle, assemble, and change media in the bioreactor, sterility problems occurred less often. We considere this hindrance solved, as the last set of experiments performed had no contamination present.

5.2 Bioreactor Capabilities Testing Using a Bioprosthetic Heart Valve

The final design of the bioreactor system is able to achieve the desired environmental conditions, but it does not reach all the goals given in the revised client statement for it's qualities as a pump. It supplied the physiological conditions of cyclic opening and closing, nutrient content, and stroke rate. However, the bioreactor system was unable to achieve a flow rate of up to 5000 mL/min or pressures reaching 120/80 mmHg. The flow rate and pressures it reached were approximately 1350 mL/min and 100/70 mmHg, respectively. These values are not quite physiological, but they are comparable to those of many other bioreactor designs. By using an alternative pumping mechanism, modifications could be made to increase these values.

Flow rate and pressure do not yet meet physiological levels, but at the current stage of tissue engineering, this is not an issue. Current tissue engineered heart valves must go through steps of progressive adaptation, with flow and pressure beginning at values much lower than those that the Clemson bioreactor can reach. As the technology improves and valves get closer to implantation, we will need to be able to reach the higher values to represent physiological conditions, but for now the current maximum values are more than adequate to for the initial stages of tissue engineered heart valve development.

5.3 Heart Valve Experimentation

The four valve experiments shown of the fifteen performed were a wide demonstration of the bioreactor's capabilities, from keeping living tissue alive, to the differentiation of stem cells. These tests adequately show the functionality of the designed heart valve bioreactor.

Many of the cells on the **living valve** were still viable after one week under static conditions and in the bioreactor. Extended experiments will help determine the different effects of static and dynamic culture on cell viability, but these results are useful to show that the bioreactor is capable of supporting cellular life in large quantities of culture media with the closest air-media interface occurring more than 20 cm away.

The **endothelial cell seeded valve** experiment was successful in many ways. Histological sections of the non-seeded valve demonstrated efficacy of the decellularization method. Endothelial cells initially attached to the valve surfaces and remained for 21 days under both static and dynamic conditions, but the differences between the cells subjected to those conditions are great. Though we were unable to quantify cell proliferation, we saw more cells present and alive in the dynamic conditions than in the static conditions after 21 days.

Live/DEAD imaging of the dynamic cusp surfaces showed cellular alignment around certain structures believed to be the large collagen bundles present in the cusp's circumferential direction. Scanning electron microscopy (SEM) confirmed and strengthened this finding, revealing some alignment on the static cusp, but notably greater alignment on the dynamic cusp. Also of note is that on the dynamic valve, the cells on

"top" of the collagen bundles were lined up in the circumferencial direction of the bundle while the cells on the base or in the "valleys" of the bundles were often lined up in the longitudinal direction of the bundles.

Perhaps the most apparent difference between the cells on the static valve with the cells on the dynamic valve was the morphology. SEM was crucial in these findings. Cells under static conditions for 21 days were often flattened and fairly dendritic. Cells under dynamic conditions for 21 days were either flattened or more rounded, but did not exhibit dendritic structures to the same extent as the cells under static conditions.

After analyzing the solution of Live/DEAD stain for the 21-day valves we found that many cells were lost during the transfer of the cusp from the staining solution to the imaging well. This suggests a greater number of cells present on the cusps than the Live/DEAD images present. It also suggests that seeded cusps are sensitive to handling and future experiments should use a "no-touch" technique for all tissue handling to prevent sloughing off of any cells.

The **adipose-derived stem cell seeded valve** showed that stem cells would remain on the cusp scaffolds for three weeks. DAPI confirmed the presence of these seeded cells on the static and dynamic valve.

Human bone marrow-derived stem cells expressed signs of differentiation after just 8 days in the bioreactor that were not present to the same extent in the static control. The cells' expression of vimentin demonstrates that the stem cells had differentiated into a fibroblast-like phenotype. Vimentin is also one of the markers used to identify valvular interstitial cells, which resemble both smooth muscle cells and fibroblasts. This implies

that the bioreactor is aiding the differentiation of these cells into cells that resemble valvular interstitial cells.

Overall, results indicated successful cell seeding and attachment in all appropriate valves, noticeable intercellular alignment in the seeded valves, and stem cell differentiation in the human bone marrow-derived stem cell seeded valve. The conditioning system provides a dynamic, three-dimensional cell culture setting to provide optimal physiological conditions for tissue engineered heart valve development over extended periods. This approach will be further developed to study multiple aspects of tissue engineered heart valve development and heart valve pathology.

CHAPTER 6: CONCLUSIONS

1) The bioreactor is adaptable for any clinically relevant valve and caused all types of heart valves to cyclically open and close.
2) Flow, pressure, and stroke rate can be controlled and reproduced in the bioreactor. Though they do not reach physiological levels, conditions of flow and pressure achieved are adequate for conditioning tissue engineered heart valves.
3) The treated surface of the decellularized, fixed, and neutralized valves was compatible with porcine aortic endothelial cells, rat adipose-derived stem cells, and human bone marrow-derived stem cells.
4) Cell viability can be maintained in the bioreactor for at least three weeks, as demsonstrated with four cell types, including native cells.
5) The bioreactor creates adequate force vectors to induce cell alignment on the cusps.
6) The bioreactor is able to induce differentiation of stem cells into fibroblast-like cells.

Results show that the bioreactor is able to provide cell-friendly conditions and create forces on the heart valve cusps, creating a controllable environment that will be necessary in the conditioning of tissue engineered heart valves.

CHAPTER 7: RECOMMENDATIONS

1) The next step that should be taken to improve this bioreactor is to increase the sample size so multiple valves can be tested concurrently. This will be important when statistically significant data is necessary. A multiple valve bioreactor should be designed in the near future.
2) Improving the flow rates and pressures is also important to be able to reach and exceed the native physiological resting conditions. A different type of pumping mechanism such as a CAM driven device might be necessary instead of a pneumatic pump.
3) An improved method of assembling the bioreactor and changing the culture media should be investigated. This would create less opportunity for intial contamination and allow the experiments to run longer with less risk of contamination.
4) A long term experiment should be performed with stem cells to examine the self-repairing capabilities of tissue engineered heart valves.

The bioreactor approach should be continually developed to study multiple aspects of tissue engineered heart valve development and heart valve pathology.

APPENDICES

Appendix A: Design Documentation

Client Questionnaire

1) How is the bioreactor to be used?
    - to test function?
    - to condition?
    - to measure mechanical properties?
    - to transport a finished product to the hospital for implantation?
    - to provide information regarding formation process of 3D tissues
    - to provide information regarding development of the cells
    - to establish a uniform distribution of cells on a 3D scaffold/facilitate cell seeding
    - other?

2) What mechanical forces will be important to measure?
    - shear stress
    - fluid speed
    - fluid density
    - pressure drop across tissue
    - total pressure magnitudes
    - pressure waveform
    - negative pressure anywhere
    - flow waveform
    - flow rate
    - stroke volume
    - frequency
    - other

3) What mechanical forces will be important to regulate?
    - shear stress
    - fluid speed
    - fluid density
    - pressure drop across tissue
    - total pressure magnitudes
    - pressure waveform
    - negative pressure anywhere
    - flow waveform
    - flow rate
    - stroke volume
    - frequency
    - other

4) How many samples would you like to run at once?

5) What is your estimate of what the cost should be?

6) What is the maximum amount of money you would like to spend, if known?

7) What types of sterilization are available on campus?

8) What nutrients will be important to measure?
   - O_2 (pO_2)
   - CO_2 (pCO_2)
   - glucose
   - temperature
   - pH
   - other

9) What nutrients will be important to regulate?
   - O_2 (pO_2)
   - CO_2 (pCO_2)
   - glucose
   - temperature
   - pH
   - other

10) How many days should the system be able to run?

11) What methods of attachment of the tissue to the bioreactor are possible or preferred?

12) How well do gasses and nutrients / wastes diffuse through the pericardium and other material used? How thick is the material to be used?

13) What will the shape of the "heart valve" be? OR: What are the sizes of the vascular grafts?
   - leaflets only
   - leaflets with some aorta
   - leaflets on a ring
   - leaflets with aorta and something on other side
   - other

14) What diameter tissues will be used

15) How much user interaction/manipulation is acceptable?
   - all computer controlled
   - adjust valves with a screwdriver
   - automatic measurements vs. computer-controlled readings

16) What type of cells or other materials will be used and what are their mechanical and chemical / nutritional requirements?

17) What are the values you would like to have available for mechanical forces?
    - embryonic conditions
    - newborn conditions
    - slow then increase in pressure and flow rate
    - values in numbers…
    - other

18) What are the values you would like to have available for nutrient levels?
    - embryonic conditions
    - newborn conditions
    - increasing with time
    - values in numbers…
    - other

19) Should the forces in the radial, circumferential, and longitudinal directions vary? If so, how?

20) Are there any other comments, wishes, or suggestions you wish to see implemented in the design of this first phase of the bioreactor? This is a chance for you to tell me what YOU want out of the bioreactor if I have missed anything in the previous questions. Please, give me input.

Attributes List

Provide physiological pressure
Provide physiological flow of nutrient medium
Be contamination-resistant
Be maximally sterile
Have adjustable pulsatile flow (50-2000 mL/min)
Have varying levels of pressure (10-240 mmHg)
Be compact
Fit inside an incubator
Provide optimal gas supply
Provide stable temperature
Provide "instantaneous" flow of fluid through material to be tested – flow waveform
Have adjustable stroke volume of ventilator
Have adjustable ventilation rate of ventilator
Expose developing tissue to "correct" physiological "signals"
Allow long term testing
Be (largely) self reliant (Utilized with additional sophisticated equipment)
Be very robust
Be easy to clean and sterilize (ethylene oxide?)
Be transparent
Allow easy attachment of valve to system
Test up to ~10 samples at one time
Provide flow conditions similar to those of natural heart valves
Establish a uniform distribution of cells on a 3D scaffold
Maintain desired concentration of gases and nutrients in culture medium
Provide efficient mass transfer to the growing tissue
Expose developing tissue to physical stimuli
Provide information regarding formation process of 3D tissues, which originate from the isolated cells
Control biomechanical environment
Control biochemical environment
Control pH
Control O_2 (pO_2)
Control CO_2 (pCO_2)
Control glucose
Be basic / not complicated / simple
Fluid flow: composition, flow rate, pressure, temperature (heat supply)
Mechanical force: pulsatile forces, pressure, flow rate, shear stress, frequency, stroke rate, stroke volume
Be adaptable to multiple cardiovascular applications
Be user friendly
Be time efficient
Have quick setup
Be repeatable

Objectives, Functions, and Constraints Lists

Objectives:

Should be compact / small
Must be very robust / durable
    Should be made of durable materials
    Machinery should be of good quality
    Must be repairable
        Must be able to separate into individual parts
        Parts must be replaceable
Should be adaptable to multiple cardiovascular applications
    Tissue-mount should be changeable for different tissues
    Tissue-mount should be adjustable for different sizes and variations of tissue
    Applied forces should be adjustable
    Nutrient / waste levels should be adjustable
Should be user friendly
    Should be basic / simple
        Should have few parts requiring multiple adjustments
        Should have few components
    Should be time efficient
        Should have quick setup
        Should have easy setup
    Should facilitate easy attachment of cardiovascular construct to the system
    Should be (largely) self reliant (little manual work by user required to change settings – screwdriver adjustments OK)
Must be repeatable
Must be sterile
    Must be sterilizible (ethylene oxide?)
    Must be contamination-resistant
    Should be transparent
    Should be easy to clean
Should be affordable to produce ($2500-$5000)

Constraints:

Must fit inside an incubator (**how of controlling temp and pH**) – means of the function
Must cost less than ~$5000
Material must not degrade in the presence of liquids

Requirements:

Vascular size changes monitored by video image capture
Minimize medium volume
Change medium in a sterile manner

Functions:

Provide information regarding formation process of 3D tissues (visually see 3D structure)
Facilitate cell seeding and establish a uniform distribution of cells on a 3D scaffold (cells may be placed on scaffold after scaffold is attached to incubator)
Test function the cardiovascular tissue (open and close heart valve or pump fluid through vascular graft)
Hold 3-6 samples at once
Condition cardiovascular tissues for both 2-3 weeks and 5-6 months (**why of maintaining sterility**)
Hold the cardiovascular tissue being tested
Display all settings and levels in a compact and understandable manner

Provide proper nutrient and waste transport to and from the tissue (pediatric conditions for valves, adult for grafts)

- Maintain temperature at 37°C (incubator)
- Maintain pH at *7.2-7.4* (incubator / CO_2)
  - Control CO_2 (pCO_2) (incubator)
- Maintain high levels of O_2 (pO_2) (~20%?)
- Control glucose levels

Apply proper forces to a cardiovascular tissue (pediatric conditions for valves, adult for grafts)

- Control pressure waveform over cardiac cycle
- Control pressure drop across tissue (160/100 mmHg for heart valve)
- Control flow waveform over cardiac cycle
- Control flow rate (50-2000 mL/min?)
  - Control stroke volume
  - Control frequency (60-70 bpm)
- Control shear stress
  - Control fluid speed
  - Control fluid density
    - Controlling fluid composition

Measure forces to cardiovascular tissue

- Pressure drop across tissue
- Pressure waveform over cardiac cycle
- Flow rate
  - Stroke volume
  - Frequency
- Shear stress
  - Fluid speed
  - Fluid density

Pair-wise Comparison Charts

Dan, Aggie, Betsy, and Tom,

On the following charts, please compare the items in the left column to each item in the top row, deciding which one is a more important quality for the bioreactor to have. These are very broad and basic goals/objectives that the bioreactor is to achieve. Some are more important than others and this will help me focus on what you believe to be the most important areas.

I included a "tree" to help you visualize which goals are sub-goals, etc.

Scoring:

Item in left column is more important than item in top row: 1
Item in left column is equally important as item in top row: 0.5
Item in left column is less important than item in top row: 0

Do not fill in the gray boxes (they are duplicates of the white boxes)

This is only to get your views on RELATIVE importance

Thank you,
Lee

Main Objectives

Objectives PCC	Compact or Small	Durable	Adaptable to Multiple Cardiovascular Applications	User Friendly	Repeatable	Sterile	Affordable to Produce	Total
Compact or Small	XXXX	0	2	1	1	1	1	6
Durable	3	XXXX	3	2	1	2	4	15
Adaptable to Multiple Cardiovascular Applications	2	0	XXXX	1	0	0	1	4
User Friendly	3	1	3	XXXX	0	2	2	11
Repeatable	3	3	4	4	XXXX	3	4	21
Sterile	3	1	3	2	1	XXXX	3	13
Affordable to Produce	3	0	3	2	0	1	XXXX	9

Repeatable
Durable
Sterile
User Friendly
Affordable
Compact
Adaptable

Sub-objective: Durable

Durable	Made of Durable Materials	High Quality Machinery	Repairable	Total
Made of Durable Materials	XXXX	1	1	2
High Quality Machinery	0	XXXX	1	1
Repairable	2	2	XXXX	4

Repairable
Durable materials
High quality machinery

Sub-sub-objective: Repairable

Repairable	Able to be separated into multiple parts	Replaceable parts	Total
Able to be separated into multiple parts	XXXX	0	0
Replaceable parts	1	XXXX	1

Replaceable parts
Able to be separated

Sub-objective: Adaptable to multiple cardiovascular applications

Adaptable to multiple cardiovascular applications	Changeable tissue-mount for different tissues	Changeable tissue-mount for different sizes and variations of tissue	Adjustable applied forces	Adjustable nutrient and waste levels	Total
Changeable tissue-mount for different tissues	XXXX	0	1	1	2
Changeable tissue-mount for different sizes and variations of tissue	3	XXXX	1	2	6
Adjustable applied forces	3	3	XXXX	3	9
Adjustable nutrient and waste levels	3	0	0	XXXX	3

Adjustable applied forces
Changeable tissue shapes and sizes
Adjustable nutrient and waste levels
Changeable tissues

Sub-objective: User friendly

User Friendly	Basic / simple	Time efficient	Facilitate easy attachment of cardiovascular tissue	(Largely) self-reliant	Total
Basic / simple	XXXX	1	0	2	3
Time efficient	2	XXXX	0	2	4
Facilitate easy attachment of cardiovascular tissue	3	2	XXXX	2	7
(Largely) self-reliant	2	2	2	XXXX	6

Easy attachment of tissue
Self reliant
Time efficient
Basic, simple

Sub-sub-objective: Basic / simple

Basic / not complicated / simple	Have few parts requiring multiple adjustments	Have few components	Total
Have few parts requiring multiple adjustments	XXXX	1	1
Have few components	1	XXXX	1

Multiple adjustments – Few components

Sub-sub-objective: Time efficient

Time efficient	Quick setup	Easy setup	Total
Quick setup	XXXX	1	1
Easy setup	2	XXXX	2

Easy setup
Quick setup

Sub-objective: Sterile

Sterile	Sterilizible	Contamination-resistant	Transparent	Easy to clean	Total
Sterilizible	XXXX	1	1	1	3
Contamination-resistant	1	XXXX	0	1	2
Transparent	1	1	XXXX	1	3
Easy to clean	1	0	0	XXXX	1

Sterilizible – Transparent
Contamination resistant
Easy to clean

Main objectives:
1) Repeatable
2) Durable
3) Sterile
4) User friendly
5) Affordable
6) Compact
7) Adaptable

Sub-objective: Durable
1) Repairable
2) Durable materials
3) High quality machinery

Sub-sub-objective: Repairable
1) Replaceable parts
2) Able to be separated

Sub-objective: Adaptable
1) Adjustable applied forces
2) Changeable tissue shapes and sizes
3) Adjustable nutrient and waste levels
4) Changeable tissues

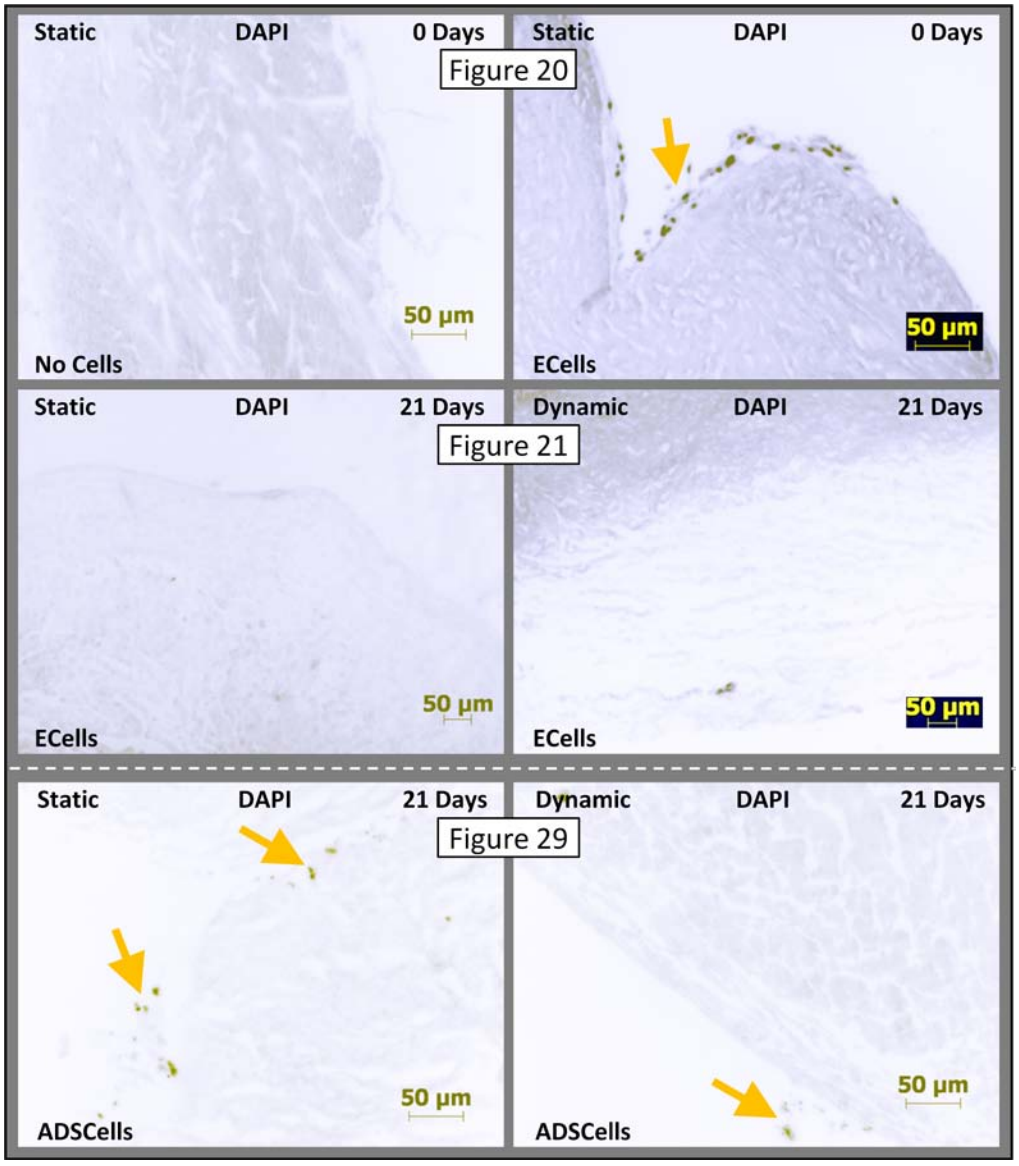
Sub-objective: User friendly
1) Easy attachment of tissue
2) Self reliant
3) Time efficient
4) Basic, simple

Sub-sub-objective: Basic / simple
1) Multiple adjustments – Few components

Sub-sub-objective: Time efficient
1) Easy setup
2) Quick setup

Sub-objective: Sterile
1) Sterilizible – Transparent
2) Contamination resistant
3) Easy to clean

Appendix B: Digitally Inverted DAPI Images



REFERENCES

Barron, V., E. Lyons, et al. (2003). "Bioreactors for Cardiovascular Cell and Tissue Growth: A Review." Ann Biomed Eng **31**(9): 1017-1030.

Bilodeau, K. and D. Mantovani (2006). "Bioreactors for tissue engineering: focus on mechanical constraints. A comparative review." Tissue Eng **12**(8): 2367-83.

Clinic, C. "Types of Valve Disease." Retrieved April 11, 2009, from http://my.clevelandclinic.org/heart/disorders/valve/valve_types.aspx#?

Darling, D. "The Internet Encyclopedia of Science." Heart Valve Disease Retrieved April 11, 2009, from http://www.daviddarling.info/encyclopedia/H/heart_valve_disease.html.

Dumont, K., J. Yperman, et al. (2002). "Design of a New Pulsatile Bioreactor for Tissue Engineered Aortic Heart Valve Formation." Artif Organs **26**(8): 710-713.

Fong, P., T. Shin'oka, et al. (2006). "The use of polymer based scaffolds in tissue-engineered heart valves." Tissue and Bio-Engineering for Congenital Cardiac Disease **21**(2): 193-199.

Freed, L. E. and G. Vunjak-Novakovic (2000). Tissue Engineering Bioreactors. Principles of Tissue Engineering**:** 143-156.

Guyton, A. C. and J. E. Hall (2006). Textbook of Medical Physiology. Philadelphia, PA, Elsevier.

Invitrogen (2005). LIVE/DEAD ® Viability/Cytotoxicity Kit *for mammalian cells*. M. Probes. Eugene, OR, Molecular Probes**:** 7.

Invitrogen (2006). DAPI Nucleic Acid Stain. M. Probes. Eugene, OR, Molecular Probes**:** 5.

LifeART. "View of an aortic valve that has been spread out showing the right, posterior, and left valves." www.fotosearch.com Retrieved April 16, 2009, from http://www.fotosearch.com/bigcomp.asp?path=LIF/LIF135/GA119002.jpg.

Martin, I., D. Wendt, et al. (2004). "The role of bioreactors in tissue engineering." Trends Biotechnol **22**(2): 80-86.

Martin, Y. and P. Vermette (2005). "Bioreactors for tissue mass culture: Design, characterization, and recent advances." Biomaterials **26**(35): 7481-7503.

Promega (2007). CellTiter 96® AQueous One Solution Cell Proliferation Assay. Instructions for Use of Products G3580, G3581, and G3582. Madison, WI, Promega**:** 12.

Rabkin, E. and F. J. Schoen (2002). "Cardiovascular tissue engineering." Cardiovasc Pathol **11**(6): 305-317.

Ratcliffe, A. and L. E. Niklason (2002). Bioreactors and bioprocessing for tissue engineering. Reparative Medicine: Growing Tissues and Organs. New York, New York Acad Sciences. **961:** 210-215.

Ross, M. H., G. I. Kaye, et al. (2003). Histology: A Text and Atlas - With Cell and Molecular Biology. Philadelphia, Lippincott Williams & Wilkins.

Schoen, F. J. (2005). "Cardiac valves and valvular pathology: update on function, disease, repair, and replacement." Cardiovascular Pathology **14**(4): 189-194.

Simionescu, D. (2006). "Artificial Heart Valves." Wiley Encyclopedia of Biomedical Engineering: 1-10.

Vesely, I. (1998). "The role of elastin in aortic valve mechanics." J Biomech **31**(2): 115-23.

Vesely, I. (2004). "Tissue Engineering of Heart Valves." Encyclopedia of Biomaterials and Biomedical Engineering: 1545-1558.

Warnock, J. N., S. Konduri, et al. (2005). "Design of a sterile organ culture system for the ex vivo study of aortic heart valves." J Biomech Eng-T ASME **127**(5): 857-861.